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Quality Assurance Guidelines for Biological Testing

Tracor Jitco, Inc, Rockville, Md

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16. ABSTRACT

This guideline document was prepared to address the need for a manual of quality assurance practices aimed specifically at biological testing. These guidelines draw from the good practices published for analytical and clinical laboratories, and incorporate observations made in a number of U.S. EPA laboratories, contractor laboratories, and biological research laboratories in general. As quality assurance aspects of biological testing depend on the particular test systems being used, these guidelines cover the general aspects of quality assurance, aquatic bioassay, microbiologic assay, and mammalian bioassay. Hopefully, attention to the principles presented in this document will assist in improving the validity and integrity of the data generated by biological testing.

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by

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FOREWORD

Protection of the environment requires effective regulatory actions which are based on sound technical and scientific information. This information must include the quantitative description and linking of pollutant sources, transport mechanisms, interactions, and resulting effects on man and his environment. Because of the complexities involved, assessment of specific pollutants in the environment requires a total systems approach which transcends the media of air, water, and land. The Environmental Monitoring and Support Laboratory-Las Vegas, contributes to the formation and enhancement of a sound integrated monitoring data base through multidisciplinary, multimedia programs designed to:

- develop and optimize systems and strategies for monitoring pollutants and their impact on the environment

- demonstrate new monitoring systems and technologies by applying them to fulfill special monitoring needs of the Agency's operating programs

In preparing these quality assurance guidelines a definite effort was made to incorporate into this one document the various aspects of quality assurance necessary to produce biological data of known quality. This required, in addition to the usual quality assurance considerations, appropriate consideration of the many peculiarities existing among those more commonly used test organisms. Considering the broad scope of this endeavor and the varied backgrounds of the expected readers, it was difficult to avoid some repetition among sections, and to determine the details necessary to meet the needs of the less experienced scientists without offending the more experienced. We believe we have minimized the repetition to the point necessary to permit each section to stand alone, and the details given are those we feel are necessary to clearly address the subject. The comprehensive references at the end of each section will permit a more in-depth coverage of any of the material presented.

George B. Morgan

George B. Morgan
Director
Environmental Monitoring and Support Laboratory
Las Vegas

PREFACE

Quality assurance is widely practiced in Biological Research and in Environmental Monitoring as in other areas of scientific and technical endeavor. However, the activity of controlling the quality of results in the subject areas is accepted as an indispensable part of laboratory management and is not usually described explicitly as a sub-discipline. In some related areas such as analytical chemistry and clinical chemistry, good quality control manuals are available. These Guidelines are intended to contribute to filling the need for a compendium of quality control practices for use in biological research.

These Guidelines draw from the good practices published by analytical and clinical laboratories and incorporate observations made in a number of EPA laboratories, contractor laboratories, and biological research laboratories in general. It was realized early and confirmed by discussions with experts in various biological fields that the quality assurance aspects of biological testing depend on the particular test systems being used. Accordingly, the Guidelines cover the general aspects of quality assurance (Sections 2 and 3.1), and then devote whole, separate sub-sections to Field Research (3.2), Aquatic Bioassay (3.3), Microbiologic Assay (3.4), and Mammalian Bioassay (3.5).

This format has led to repetition of some concepts many times. However, the user with a particular interest in one field of bioassay needs to refer only to the general sections and to that part of the rest of the Guidelines appropriate to his field.

Recognition is given to the assistance given by many laboratories of the Office of Research and Development and by some of their contractors. This is a first endeavor at bringing together in one place the good practices observed in many laboratories, confirmed by experience, and gleaned from the literature. With time and use, the Guidelines should help in maintaining the validity and integrity of data derived from biological testing.

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SECTION 1

INTRODUCTION

1.1 PURPOSE OF THE QUALITY ASSURANCE GUIDELINES

The purpose of the Quality Assurance Guidelines is to provide concepts and methodologies which can be used to maintain and improve the quality of data in laboratory and field investigations. It is intended to provide information needed for the development of quality control plans adapted to the data needs of a wide variety of programs in biological research.

The essential characteristics of data of quality are validity and integrity.

1.1.1 Valid Data

By valid data we mean data supported by objective truth. That means data from a well-planned experiment, obtained using standard methods of test and employing instruments or observational techniques which have acceptable performance. Acceptable performance implies measurement systems (method plus instrument) which have specificity, have sufficient sensitivity, precision, and accuracy for their intended use, and are practical.

Specificity requires that the test actually measure the property of interest. It also means that the test data reflect as little as possible the effects of interferences. Thus it applies to qualitative properties of the substance being measured. Specificity is an inherent property of the method, and it should be investigated before the method is adopted for regular use. We mention it here because of its implications for quality data but will not discuss it further because methods development is outside of the scope of these Guidelines.

Sensitivity refers to the ability to detect differences in the quantity of a substance in a specimen or to make a yes or no judgment regarding the occurrence of an effect. The smaller the amount to be detected, the more sensitive the test must be. However, it is not prudent to have a system more sensitive than required. Sensitivity is a judgmental requirement that must be assessed, usually at the time the method is being developed or the instrument is being acquired, so it will not be discussed further in these Guidelines.

Precision is the degree of agreement among repeated measurements made using a constant measurement system. The term may also be used to mean the degree of agreement among repetitions of an experiment. Precision is usually expressed in terms of a multiple (usually 2, corresponding to 95% probability) of the standard deviation of the measurements - the smaller the standard deviation, the better the precision. It is stated, in the units of measurement, as a plus and minus spread around the reported value. The reported value may be an individual measurement or an average. See Figure 1.1 (a).

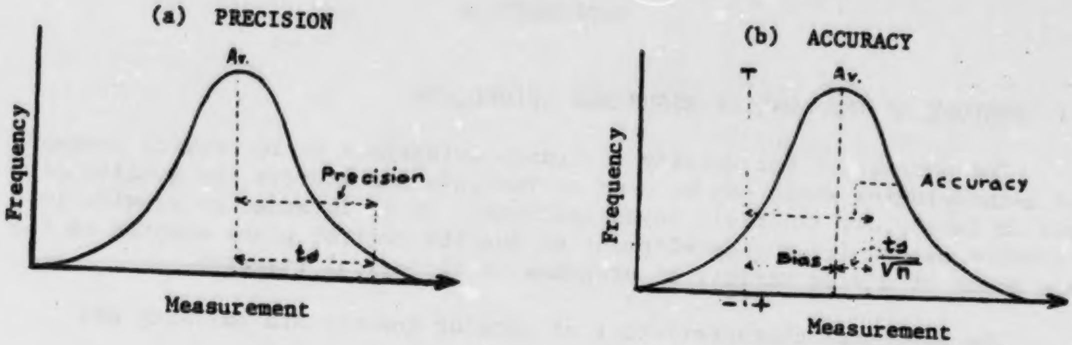

Figure 1.1 Precision and Accuracy

Accuracy is a measure of the difference between the average of a set of measurements and a standard or known value. It is expressed in the units of measurement, as a distance between the average and the known value. Thus, loosely defined, it is synonymous with the often used terms constant error, or bias, and applies to the average, not to individual values. This concept of accuracy, though sometimes used, is not completely satisfactory from the statistical viewpoint because there is always some uncertainty in the determination of the average. It is preferable to add to the bias the precision of the average, as illustrated in Figure 1.1 (b).

By practicality of the measurement system we mean that the data have been generated with appropriate speed, at reasonable cost, by methods requiring achievable technical skills and that are dependable and safe.

1.1.2 Integrity

By integrity of the data is meant that it is complete and undivided, that the information originally sought has in fact been gathered, and that measurements have not been altered or lost either by conscious action or by carelessness. The maintenance of integrity depends on control over the performance of the experiment, adherence to the detail of the measurement process, and careful handling of the data. Errors can arise during handling of the data due to transcription, clerical, or typing mistakes as a result of the use of different statistical methods at different times, computer mistakes or omissions, inclusion of the wrong data, omission of parts of the original data, differences in observational results (as between two pathologists) and changes in interpretation of the data.

These desirable characteristics of data are central to the purpose of the Quality Assurance Guidelines. It is for the purpose of achieving quality data that we shall cover standardization of methods, calibration of instruments, statistical quality control, sampling, design of experiments, data handling, training, supervision and all the other elements of a quality assurance program.

Underlying the requirement for Quality Assurance is the necessity that data be scientifically verifiable and that they stand up in court if the results of research are questioned.

1.2 DEFINITIONS

1.2.1 Quality Assurance

Quality assurance is defined as all those activities which contribute to producing correct and reliable data. Personnel assignment, facilities design, methods development and equipment selection are all important. However, in these Guidelines, emphasis will be on methodology in standardization, control, and audit of performance of work. This is consistent with the basic concept that quality control means making the best use of resources available. Efforts are measured and if results are not within acceptable limits personnel must be retrained, facilities and equipment must be upgraded, or methods improved.

The quality assurance program is developed to minimize the variations that are inherent in all research and testing. Standard operating procedures and statistical techniques are used to identify and control assignable causes of variation. Random errors are measured and used to express the degree of confidence to be placed in results. The total quality assurance program is rounded out by regular assessment by program managers of the degree of success in standardization and control.

Standardization may appear to be too harsh a concept to be applied to research. However, it is needed to assure that the work will meet the first requirement of good science, namely that it can be repeated and the results verified by other scientists.

1.2.2 Biological Research

Biological research is defined as all types of experimentation in which the test subject is a form of life. In general, however, biological research concerned with the environment is performed in non-clinical laboratories. Clinical laboratories are understood to be medical laboratories engaged in the direct examination of the condition of human patients. Non-clinical laboratories are confined almost entirely to the use of non-human subjects. There are exceptions, such as experimentation with human cell cultures, certain host-mediated assays, and in the health effects area. There has been a great deal of progress in quality control in clinical chemistry, and what is applicable to biological research, as we define it, has been adapted.

Biological research is supported by analytical chemistry. Analytical chemistry is another area in which there has been much progress in quality control. The Quality Assurance Elements in the following Section 2 are based very largely on experience gained in the analytical quality control field. We begin to build on the analytical base, and in later sections of the Guidelines, devoted to particular areas of biological research, we attempt to bring the user of the Guidelines up with the state of the art in quality assurance of biological research.

1.2.2.1 Laboratory Research--

It is convenient, because of the different degree of attention required to sampling, testing, and control of the experiment between work done in the laboratory and in the field, to make a distinction between laboratory and field research.

Laboratory research is that research done in a fixed laboratory location equipped with all supporting services and usually environmentally controlled.

1.2.2.2 Field Research--

Field research is research done under field conditions, usually with test subjects in a feral state. Testing equipment may be deployed in the field or may be located in mobile laboratories more or less equipped and environmentally controlled.

Controlled research is defined as a field research, in which constraints are placed on test subjects, test environment, and/or application of treatments. Examples are chemical treatment of algae in some areas of a pond, or treatment of fish in a confined area of a stream with measured doses of a chemical.

Effluent observations are defined as research in which existing contaminant levels and condition of test subjects are measured as they are found in the field.

SECTION 2

QUALITY ASSURANCE ELEMENTS

2.1 QUALITY ASSURANCE POLICY AND OBJECTIVES

Every laboratory and field organization should have a clearly enunciated policy regarding quality of data. This should include a statement by management of its concern for quality. The purpose of a statement of quality policy by management is to ascertain that quality control is a pervasive concern; one that merits attention not only at critical points, but daily in the routine performance of research. The statement by top management to the laboratory must be followed up with continuing visible evidence of its sincerity to all levels of the organization. Periodic meetings should be held in the laboratory to discuss quality objectives and progress toward their achievement.

Points for a quality policy and corresponding quality assurance objectives are given in Table 2.1. The objectives are spelled out in more detail in the following paragraphs. Appropriate methodologies for the attainment of the objectives are given in the referenced Sections.

2.1.1 Laboratory Evaluation

Laboratory evaluation is widely practiced as a basis for certification or accreditation of laboratories. For example, in compliance with the Safe Drinking Water Act, EPA has a State Laboratory Certification Program (Geldreich, 1975). Evaluation, whether carried out by outsiders or by the laboratory itself, can be a useful management tool for improvement. Such an evaluation technique is available for environmental monitoring laboratories (U.S. EPA, 1978). This procedure covers personnel, laboratory space and facilities, analytical methodology, analytical instruments and apparatus, and quality control, including interlaboratory testing.

Adaptions of the latter procedure for use in biological research laboratories would include a number of features unique to that kind of laboratory. In the facilities area, there are requirements for separation of clean and dirty corridors and separate rooms for isolation of test subjects by species and of test materials, at least by class. Acceptable animal care standards are implied and also appropriate experimental apparatus and techniques. The design of bioassay experiments would be covered. Proficiency testing, i.e., the submission of blind samples to the laboratory and taking scores on the tests into consideration in the evaluation, is accepted as an integral part of a sufficient evaluation procedure. In biological research such testing is relatively infrequent and needs further attention. Proficiency testing can be continued between periodic evaluations as a means of interlaboratory comparison.

TABLE 2.1 QUALITY POLICIES AND OBJECTIVES FOR BIOLOGICAL RESEARCH

	Policy	Q.A. Objectives	Section
1	To provide adequate personnel, facilities and equipment	Use laboratory evaluation as a management tool	2.1.1
		Organize for quality	2.1.2
		Train for quality	2.1.3
2	To develop and use rugged methods of experimentation, sampling and testing	Use statistical consultation in design of experiments	2.2
		Apply formal sampling plans	2.3
		Measure and control precision and accuracy of tests	2.4
3	To provide adequate support for a Quality Assurance Program	Maintain good housekeeping and laboratory services	2.5
		Control test materials, chemicals and reagents	2.6
		Control test subjects	2.7
4	To demonstrate good control of research and monitoring	Use good supervisory practices to assure that protocols are followed	2.8
		Use care in preparation of materials for measurement	
		Control measurements and take action to correct deficiencies	
		Preserve integrity of data and provide adequate computer support	
5	To improve laboratory capabilities continuously	Participate in interlaboratory testing program	2.9
6	To produce reliable data and reports	Use statistical expertise in analysis of results	2.10
		Establish regular audits of performance	
		Adopt a system for review and publication of reports	

2.1.2 Organization for Quality

Quality is a concern of the whole laboratory and responsibility for the control of quality is shared by all levels of organization in accordance with their capabilities to contribute to it effectively. For example, management must set the tone by clear enunciation of quality policy and goals and support of their attainment by giving adequate attention to facilities, equipment, personnel competence, standards, operating practices, quality control programs, and performance reviews. Study directors must plan, assemble appropriate equipment, select methods, instruct researchers, monitor and adjust performance and check results.

Scientists and technicians must follow study protocols, use approved methods, apply appropriate quality control procedures, maintain chain of custody of test materials and test subjects, preserve the integrity of data, and use good scientific judgment. Supporting staff, such as analytical laboratories, consulting statisticians, or quality control specialists, must assist the whole organization to the extent that such expertise is not available within the study group itself.

Many of the activities identified as elements of quality control are widely recognized as good laboratory practices. Some of these practices are honored by time, and experienced researchers may be expected to follow them conscientiously. Some of them, of which statistical design and analysis of experiments may be an example, have been a part of the academic training of some scientific disciplines only in recent years.

The direct control which the study director may have over daily routines may be diluted by the size of the programs or by commendable delegation of responsibility to junior scientists or technicians. He also depends on analysts and other experts for support. Quality control procedures are as much directed toward coordination of a multiplicity of activities as they are toward providing safeguards against human fallibility.

We look upon quality control as a self-discipline, by the individual and the groups of individuals who conduct a study or contribute to a laboratory program. Quality control emphasizes the validity and integrity of data not for the purpose of constraining research but to enhance verifiability by the scientific community and credibility should the data be contested in the courts. It supplies the whole study or the whole laboratory with a discipline which is complementary to the scientific discipline of a good study director or researcher.

Responsibility for quality is shared by the entire organization. However, to make any plan go, it is necessary to have a leader. In large organizations, leadership may be assigned to a Quality Control Department with well-defined authority. In smaller laboratories, quality may be a clearly defined part of the job of all Section Chiefs, or of a Quality Control Committee. Better than a committee may be a part-time Quality Control Coordinator who must have sufficient authority to see to it that the laboratory's quality objectives are being met.

2.1.3 Training for Quality

The people who have an impact on quality (bench researchers, supervisors, etc.) should all be trained in the reasons for the benefits of standards of quality and the methods by which high quality can be achieved. This may be on-the-job training for most laboratory personnel but those with assigned responsibility for leadership in the quality control program should receive formal training in modern methods of statistical quality control.

Training courses are offered regularly by the Education and Training Institute of the American Society for Quality Control, 161 W. Wisconsin Avenue, Milwaukee, Wisconsin 53203, and by local sections of the Society. Also full-term courses are offered by many universities.

2.1.4 Other Objectivies of a Quality Assurance Plan

In biological research, approval of all study protocols prior to their initiation should be required. The approval procedure may consist of a peer review and a review by the various supervisory levels. It should also require comment by a statistician on the design of the experiment and statistical analysis to be used. The statistician must be consulted early in the planning stages to assure that the design meets requirements of statistical adequacy and that methods of statistical analysis are specified.

Because of the high level of variability of biological materials, special attention should be given to sampling. A sampling procedure may have to be designed, much in the way that an experiment has to be designed. Formal sampling requires attention of a statistician who can assist in determination of location and frequency of sampling as well as the size and number of increments needed in the sample. A chain of custody should be established to control the flow of samples from field, through the laboratory, to storage and eventual disposition.

The need for close supervision, particularly of long-term chronic experiments and wide-flung monitoring activities, has recently emerged as an important problem. The quality assurance plan should be an arm of the supervisor in accomplishing the aim of producing quality data. Brief, timely quality reports are one means of keeping the supervisor advised. There should be a good bookkeeping system particularly for collecting observations made during the conduct of the experiment. These observations should be made on a suitable schedule which is frequently oftener than daily. The observations should be assembled frequently, analyzed (by computer if necessary) and reported promptly to highlight problem areas. In addition, the supervisor should be close enough to the operation of the laboratory to be sure that procedures are being followed as intended and that the observations are being correctly assembled.

The laboratory should have a written plan including the quality assurance procedures detailed in the remaining parts of Section 2. As part of the plan, all the documentation should be assembled in a Quality Control Program Manual. This manual should be available for use in the laboratory and as a basis for evaluation of the laboratory's performance in accordance with the plan.

Section 2 covers the general aspects of quality assurance methodology - those parts applying to any type of biological research. Following Section 2 are specialized Sections which describe methodologies required to meet the requirements of particular areas of research.

2.2 DESIGN AND ANALYSIS OF EXPERIMENTS

2.2.1 Description of Design of Experiments

The design of experiments is that part of research planning that has to do with precise scoping of the work to be done. This involves the layout of the number of levels of treatments, number of test subjects per treatment group, use of controls, replications (repetitions of treatments), duration of treatment, identification of test materials and test subjects, route of administration, the response to be measured, and description of special circumstances surrounding the experiment.

The design work can be done most effectively if experience is available from earlier experiments and if done with full attention given to the implications of the design for later statistical analysis. Therefore, the design of the experiment should involve joint efforts on the part of the experimenter and a statistical consultant.

The statistician should be involved, along with the experimenter, in selection of number and levels of treatment, number of test subjects per treatment, and the use of controls and replication of treatments. These activities influence the selection of the proper mathematical model of the experiment, the measurement of experimental error, and the significance (in terms of probability) that can be attached to results. The other activities including duration of treatment, identification of the response to be measured and the test method to be used, selection of test materials and test subjects, and route of administration are the prerogative of the experimenter.

The subject of design of experiments owes much to the work of two men, Ronald A. Fisher and Frank Yates, through work at Rothamsted Experimental Station since its founding in 1920. Thus, much of the subject has grown through its use in an experimental science. This happens to have been in biological science, largely agricultural at first, but rapidly expanded into genetics and all kinds of bioassay.

Fisher's Design of Experiments (1947) and Yates' Design and Analysis of Factorial Experiments (1937) are classics in the statistical literature. They have been followed by Cochran and Cox's Experimental Designs (1950), Finney's Statistical Method in Bioassay (1964), Kempthorne's Design and Analysis of Experiments (1952) and others. Most of the complex experimental designs now available for survey work or in scientific/technical experimentation have grown from the pioneering work done in the biological area.

Great care should be exercised in designing biological experiments because the wide scope of biological experimentation, the special methods of test and observation, the responses measured, the variability of test subjects, the complexity of biological theory, and the difficulty in standardizing designs all lead to problems that require special attention.

The instances in which the needs of the experimenter are satisfied entirely by an experimental plan, the analysis of variance (a widely used statistical method) and standard errors, are comparatively few. What is needed is a broad understanding of experiment design and relation of this subject to the general theory of statistics and to the problem of experimental inference.

The distinguishing feature of science is its method. The central thrust of scientific method is examination of what is known, and the formulation therefrom of hypotheses which can be put to experimental test. The word "experimental" is the most important one, and therefore the design of experiment appears as the crux of scientific method.

The determination of the relevant aspects of the problem for which a solution is required and the actual formulation of the hypotheses to be tested require attention of the keenest sort. The experimenter must be very knowledgeable in his biological field. After formulation of the hypotheses comes consideration of consequences that are verifiable and, finally, objective verification. Here is where the intuition or genius of the experimenter can be enhanced by help of the statistician.

Verification of a hypothesis cannot be absolute. It can only be shown that observations made are compatible with the theory within the limits of error to which the observations are subjected. In other words, it is possible only to prove that a hypothesis is false, thus, the use of the null hypothesis in statistics.

The scientific method is circular, proceeding from observation through abstracting of essential information as a basis for a logical theory, development of the theory, and prediction of new events, back to observation and through the cycle again. Statistics enter at the taking of observations and at the comparison of the observations with predictions from theory.

It is essential that the hypotheses and their possible outcomes be formulated before verification is attempted. Hypotheses formulated from or modified by the observations are suspect. It is one of the basic notions of statistics that probability statements cannot be made about statistical tests suggested by the data to which they are applied. Therefore, selection of the statistical methods to be applied must be made before the experimental work is carried out.

The design of an experiment is the pattern of the observations to be collected. There are two types of experiments: absolute and comparative. In an absolute experiment, repeated observations, which do not agree exactly with each other, are made on a test subject to obtain the best estimate of some property of the subject and a measure of the reliability of the estimate. A sample survey is an example of an absolute experiment for determining particular characteristics of a population.

In a comparative experiment, the outcomes of two or more treatments are compared in their effects on characteristics of a population. This requires taking of controlled observations, where control is effected on all treatments to the same degree by either fixing all the variables in the experiment or controlling them statistically by randomization.

The economic aspect of experimentation must be emphasized. The experimenter is usually in the position of being able to spend only a certain amount of time, effort and money on his investigations. There are more efficient and less efficient ways in which he can go about the work, leading to greater or lesser degrees of certainty in his results. He must consider the cost of obtaining a given level of certainty, whether it is worth the cost, and at what stage the cost of increased certainty is too great.

2.2.2 Steps in the Design of Experiments

A statistically designed experiment consists of the following steps (Kempthorne, 1952):

- Statement of the problem
- Formulation of hypotheses
- Devising of experimental technique and design
- Examination of possible outcomes and reference back to the reasons for the inquiry to be sure the experiment provides the required information and does so to an adequate extent
- Consideration of the possible results from the point of view of the statistical procedures which will be applied to them, to ensure that the conditions necessary for these procedures to be valid are satisfied
- Performance of experiment
- Application of statistical techniques to the experimental results
- Drawing conclusions with measures of the certainty of estimates of any quantities that are evaluated, careful consideration being given to the validity of the conclusion for the population of subjects to which they are to apply
- Evaluation of the whole investigation, particularly by comparing it with other investigations of the same or similar problems

A check list of the detailed activities required in carrying out these steps is given in Appendix A.

2.2.3 Essential Statistical Concepts

It is not intended here, or in Section 3.4.7 on Statistical Analysis, to give a complete description of statistical theory. Some familiarity with statistics on the part of the experimenter is assumed. The requirements for statistical theory which go beyond what can be expected of the average experimenter are the reason for recommending that statistical advice be sought at the very beginning of planning an experiment.

It is worthwhile to consider, briefly, the elementary statistical concepts that are essential to the design and analysis of experiments.

The first concept is that of a population. A population is an assemblage of the objects of possible observation or measurement, or some attribute of those objects. The individual objects in the population may be arranged according to the size of a measurable characteristic, and the function giving the relative frequency of the individual measurement is called the distribution of the individual objects. From this distribution, we may obtain the proportion of measurements less than a chosen value, or the proportion lying in any chosen interval of values. A distribution may be continuous or discrete: for example, the ppm bioconcentrations of a toxic substance in test objects under treatment will be continuous or variable measurements, whereas counts of the number of neoplastic lesions under a treatment will be integers, with a discrete distribution.

Other kinds of discrete measurements include ranks (observations ordered according to magnitude), or attributes (yes or no responses to a treatment).

2.2.3.1 Normal Distribution Statistics--

Distribution curves should be familiar to users of the Guidelines, for example, the symmetrical bell shaped curve for the normal distribution illustrated in Figure 2.1.

The most useful measure of central tendency of a distribution is its average (mean), μ. The measure of spread of a distribution most generally used is the variance, σ^2, which is the mean square distance of the population individuals from the average.

The median, M, is a measure of location useful in biological research. It is the measurement in an ordered array that has an equal number of measurements on either side of it (it divides the distribution in half). The median would be preferred over the average, for example, if an animal behaviorist is studying the time from the beginning of an experiment until each individual responds. He can obtain the median time of performance without waiting for all the animals to respond and then calculating an average. Thus, if the experimenter knows what the total sample size is, he can get an estimate of the central tendency without waiting for slowest responders. Moreover, some may never respond, so calculation of a true average may be impossible.

A distribution is characterized by a mathematical form containing quantities called parameters which, when known, describe the distribution completely. The estimation of the parameters from sample data is one of the most important functions of statistical theory.

The most used distribution is the normal distribution which has the advantage that the average, and standard deviation (square root of the variance, or root mean square deviation), σ, describe it completely. The quantity that best estimates the average of a population from a random sample size n, is the average of the sample, $\bar{x}$, and this estimate has a variance equal to the variance of the individual measurements divided by n.

The estimate of the variance, s^2, is $[1/(n-1)]\Sigma(x-\bar{x})^2$, where n is the sample size, x is an individual measurement, and $\bar{x}$ is the sample average.

A test of significance of a sample average (of its difference from the population average, or "true" value) is based on Student's distribution where:

$$t = (\bar{x} - \mu) / (s/\sqrt{n}) \qquad \text{Eq. 2.2.1}$$

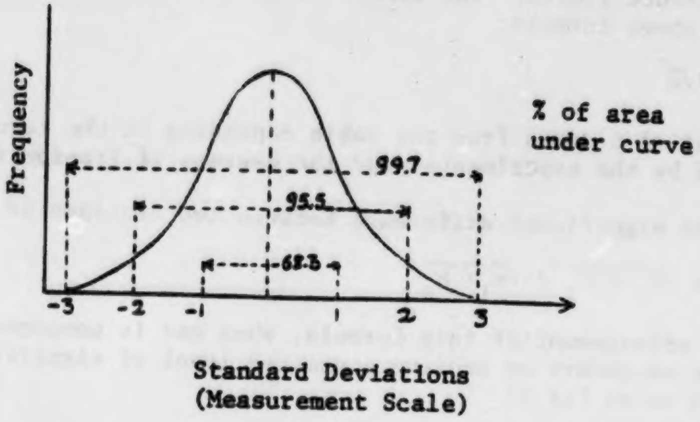

Figure 2.1 Normal distribution.

The value that t takes for any level of significance and sample size (actually for n-1, the degree of freedom) may be looked up in a table (Fisher and Yates, 1949), or in most statistical texts.

The value of t is the number of standard deviations in the difference being tested, small sample size being taken into account: t is given for various significance levels. As the sample size increases, the distribution of t approaches the normal distribution. The relationship between the number of standard deviations and the significance level (area under the normal curve) for the normal distribution is shown in Figure 2.1. Significance levels are usually expressed for even proportions, such as 95% (1.96, say 2.0 standard deviations) or even standard deviations such as 3.0 (99.73%, or practical certainty).

The numerator in the formula for t may be the deviation of the average from any expected value, for example, from zero, or from the known value of a spiked sample.

To test the significance of the difference between the two averages, the formula becomes

$$t = [(\bar{x}_1 - \bar{x}_2) / s] \sqrt{(n_1 \times n_2) / (n_1 + n_2)} \qquad \text{Eq. 2.2.2}$$

where the subscripts identify the two sets of numbers and s^2 is the pooled variance of the two sets, which has $n_1 + n_2 - 2$ degrees of freedom.

Of perhaps even wider application than tests of significance is the usage of confidence limits. The limits on either side of the sample average are, from the above formula,

$$\pm L = ts/\sqrt{n} \qquad \text{Eq. 2.2.3}$$

where the value of t comes from the table depending on the level of significance selected by the experimenter and the degrees of freedom in the sample.

The least significant difference between two averages is given by

$$\text{LSD} = ts\sqrt{n_1 + n_2} \,/\, \sqrt{n_1 \times n_2} \qquad \text{Eq. 2.2.4}$$

Another arrangement of this formula, when one is concerned with the size of sample necessary to achieve a desired level of significance in an average, is to solve for n:

$$n = (ts/D)^2 \qquad \text{Eq. 2.2.5}$$

where s is an estimate of the standard deviation from early data and D is the allowable difference between the average of the sample and its "true" (population) value.

The economic aspect of experimentation has been mentioned earlier. In a statistical sense, the value of a better experiment is determined by the ability to predict a result of one or several treatments with greater precision. Another measure is that of the quantity of information, for which Fisher (1925) suggested $nI = n/s^2$, from which it derives that the information per observation is $1/s^2$. The economic experimenter, therefore, increases n within limits of resources and reduces s by use of sound experimental design, precise instrumentation or careful observation, and meticulous supervision of the conduct of the work.

2.2.3.2 Statistics of Other Distributions--

The test for the significance of differences between two sample variances or the differences of means of several samples is the F test:

$$F = s_1^{\,2} / s_2^{\,2} \qquad \text{Eq. 2.2.6}$$

The degrees of freedom are $n_1 - 1$ and $n_2 - 1$. This test is used in the analysis of variance of designed experiments.

Standard values of F may be looked up in standard statistical tables. Several variances may be compared by Bartlett's test.

The χ^2 (Chi-square) test is applied to problems in which we wish to determine whether the frequency with which an event has occurred is significantly different from that which was expected.

$$\text{Chi-square} = \Sigma\,(O - E)^2 / E \qquad \text{Eq. 2.2.7}$$

where O is the observed frequency in a group and E is the expected frequency. The degrees of freedom are the number of groups minus 1. The standard values of Chi-square are found in the standard statistical tables.

Discrete experimental data frequently conform to the binomial, Poisson, or negative binomial distribution. The binomial is the distribution of the number of observations of either a yes or no character (say morbid or healthy animals following a treatment) in n trials. The chance of a favorable observation (success) is p. Then the estimate of the average of the distribution is p and the variance is pq (where q is equal to $1 - p$). When n is very large the binomial approaches the normal distribution. The t test for significance of an average portion is:

$$t = (p - k) / \sqrt{pq/n} \qquad \text{Eq. 2.2.8}$$

where k is some desired proportion. The formula for sample size is:

$$n = t^2pq / D^2 \qquad \text{Eq. 2.2.9}$$

The Poisson distribution provides probabilities of the number of observations per unit of time, area, volume, etc., for example, the number of bacterial colonies per unit area or volume of a culture. The average count and the variance are the same, c.

The t test for significance of a count per unit is:

$$t = (c - m) / \sqrt{c/n} \qquad \text{Eq. 2.2.10}$$

where m is some desired count. The formula for sample size is:

$$n = t^2c/D^2 \qquad \text{Eq. 2.2.11}$$

The negative binomial distribution is applicable because of clustering (or contagion) of "successes" of an otherwise binomial distribution, for example, deaths of insects. An example of its application to biological research is given in Bliss and Fisher (1953).

It is not correct to treat data from these discrete distributions as though they were normal. Many of the commonly used analytical methods such as the analysis of variance, are based on a number of assumptions.

Among the assumptions underlying the use of the analysis of variance are:

- The sampling of individual items must be at random
- The experimental error must be a normal random variable (the individual measurements must be independent)
- The variances in groups of samples must be equal
- Effects of treatments must be additive (if interactions are present they must be taken into account).

If these assumptions cannot be maintained, it may be possible to use a distribution-free test. Tests based on ranking of the measurements include the Kruskal - Wallis test, the Mann - Whitney U-test, and the Wilcoxon two-sample test. See Sokal and Rohlf (1969). Other tests include nonparametric multiple comparisons by STP, Friedman's method for randomized blocks, and Wilcoxon's signed-rank test for two groups (Ibid.).

2.2.3.3 Data Transformation--

The measurements to be analyzed may frequently be transformed to meet the assumption of the analysis. The entire analysis can then be carried out on the transformed measurements. A fortunate fact about transformation is that very often several departures from basic assumptions are cured simultaneously by the same transformation to a new scale. When a transformation is applied, tests of significance are performed on the transformed data but estimates of the averages are usually reported in the original scale.

The most common transformation is conversion of the measurements into common logarithms. This transformation is useful in studying the growth of organisms.

When the data are counts, such as of blood cells in a hemocytometer, the square root transformation is frequently useful. Such data follow the Poisson distribution where the variance equals the mean. Transformation makes the variances independent of the means.

The arcsine transformation is especially appropriate to percentages and proportions where, for example, the measurement may be the percent fertile in a vial of eggs of Drosophila.

2.2.4 Experimental Models--

The simplest possible experiment is application of a single treatment to a group of two or more objects, for which the framework is

Treatment with a Toxic Material

Animal 1
Animal 2
.
.
Animal n

A simple linear expression provides an analytical model for this experiment: $y = \mu + T + e$.

The meaning of this model is that a single measurement, y, can be decomposed into the average, a fixed deviation of the measurement from the average (T) and a random deviation of the measurement from its expectation (e) which is $\mu + T$.

Analyses possible with this model include:

Before the experiment
- Calculation of the number of animals required to estimate the average within desired limits (only if a prior estimate of the variance is available). See Section 2.3, Equations 2.3.1 and 2.3.5.

After the experiment
- Significance of the average. Eq. 2.2.1
- Confidence limits for the average. Eq. 2.2.3
- Sample size for further experimentation. Eq. 2.2.5; 2.2.9; 2.2.11

There are two models of the analysis of variance, as first defined by Eisenhart (1947). In Model I, it is assumed that the differences among group averages are due to fixed treatment levels. The purpose of the experiment is to estimate the true differences among the group averages.

The basic form of Model I is given by: $y_{ij} = \mu + T_i + e_{j(i)}$ where i takes values from 1 to m, the number of treatments, and j takes values from 1 to n, the number of individual objects per treatment group. The parentheses about i read "j's random within the i's."

Examples of Model I in biological research include treatment of groups of animals with different concentrations of a toxic substance. The model also fits exposure of plants to different levels of stimulant or culture of bottles of insects at different temperatures. Another example is comparison of the body weights of several age groups of animals.

The design framework is:

Treatment		
Level 1	Level 2	Level m
Animal 1.1	Animal 2.1	Animal m.1
Animal 1.2	Animal 2.2	Animal m.2
:	:	:
Animal 1.n	Animal 2.n	Animal m.n

Model II assumes that in place of fixed treatments there are randomly selected treatments different for each group. The basic form for Model II is: $y_{ij} = \mu + T_{(i)} + e_{(ij)}$, where, again, the parentheses indicate randomness. An example is the determination of DNA content of rat liver cells from three preparations from the liver of each of five rats: $m = 5$ and $n = 3$. The rats were selected at random and the preparations were made from aliquot portions of the livers.

Design Framework

	Rat Liver 1	2	3	4	5
Preparation	1.1	2.1	3.1	4.1	5.1
	1.2	2.2	3.2	4.2	5.2
	1.3	2.3	3.3	4.3	5.3

Both of the models presented above are single factors with replication. In experiments involving two or more factors, the models may be mixed, having both fixed and random factors. Obviously, no measure of experimental error is provided without replication. In two-factor or larger experiments, it is possible to use higher order interactions in place of error to test the significance. Also, in mixed models, the replication error may not be the proper denominator in the F-test. However, it is recommended that plans for replication be included in all biological experiment designs.

The variance tables for the two single-factor models look the same although there is a formal difference in the estimation of expected mean squares for treatments and the hypotheses tested are stated differently. The variance table for a single factor with replication is:

Variance Table

Source	Sum of Squares	Degrees of Freedom	Mean Squares	Expected MS	F Test
T	SS_1	$m - 1$	$SS_1/(m - 1)$	$\sigma^2_e + n\sigma^2_T$	MS_T / MS_e
e	SS_2	$m(n - 1)$	$SS_2/m(n - 1)$	σ^2_e	
Totals	SS_T	$mn - 1$			

$$SS_1 = \Sigma^m (\Sigma^n x)^2 / n - (\Sigma^m \Sigma^n x)^2 / mn$$

$$SS_2 = SS_3 - SS_1$$

$$SS_T = \Sigma^m \Sigma^n x^2 - (\Sigma^m \Sigma^n x)^2 / mn$$

The computational methods required may be found in almost any statistical textbook or a computer program may be used.

In Model I the hypothesis tested is: $H_0 : T_i = 0$

In Model II: $H_0 : \sigma^2_T = 0$

One-factor models with replication are sometimes described as between-and within-group models, and the mean squares are designated as between and within variances, s_b^2 and s_w^2, respectively.

Experiments may involve two or more factors and may involve mixed, fixed, or random factors. Also these factorial designs may be supplemented by many available random blocks, splitplots, square designs, nested designs, response surface designs, and others. These experiments are adaptable to both qualitative and quantitative factors and the analytical methods used when the experimental results are in must depend on this and on the nature of the responses measured or observed. Here again, the need for a good statistician to assist with the data analysis is obvious.

Most biological research may involve single-factor experiments where all factors but the treatments of interest are controlled by balancing or randomization. However, in the interest of obtaining most information from experiments, it may be possible in many experiments, with slight additional attention to the structure of the experiments, to use more complex models effectively.

The general models for two factors are:

Fixed Factor: $y_{ii}k = \mu + A_i + B_j + AB_{ij} + e_{kij}$, where AB is the interaction of the two factors.

Random Factor: $y_{ijk} = \mu + A_i + B_{ij} + e_{ijk}$

2 Factors, Fixed, with Replication

		Factor A			
		Level 1	Level 2	. . .	Level m
	Level 1	Animal 1.1.1	Animal 2.1.1		Animal m.1.1
		⋮	⋮		⋮
		Animal 1.1.n	Animal 2.2.n		Animal m.1.n
Factor B	Level 2	Animal 1.2.1	Animal 2.2.1		Animal m.2.1
		⋮	⋮		⋮
		Animal 1.2.n	Animal 2.2.n		Animal m.2.n
	Level r	Animal 1.r.1	Animal 2.r.1		Animal m.r.1
		⋮	⋮		⋮
		Animal 1.r.n	Animal 2.r.n		Animal m.r.n

Variance Table

Source	S.S.*	D.F.	M.S.	E.M.S.	F
A	SS_1	$m - 1$	SS/DF	$\sigma^2_e + n\sigma^2_{AB} + nr\sigma^2_A$	MSA/MSAB
B	SS_2	$r - 1$	SS/DF	$\sigma^2_e + n\sigma^2_{AB} + nm\sigma^2_B$	MSB/MSAB
AB	SS_3	$(m-1)(r-1)$	SS/DF	$\sigma^2_e + n\sigma^2_{AB}$	MSAB/MSE
e	SS_4	$mr(n-1)$	SS/DF	σ^2_e	
Totals	SS_T	$mrn - 1$			

2 Factors, Random

Factor A

	Level 1			Level m		
Factor B	**Level 1**	Level 2 ...	Level r	Level 1	Level 2 ...	Lever r
	Animal 1.1.1	1.2.1	1.r.1	m.1.1	m.2.1	m.r.1
	⋮	⋮	⋮	⋮	⋮	⋮
	Animal 1.1.n	1.2.n	1.r.n	m.1.n	m.2.n	m.r.n

Variance Table

Source	S.S.	D.F.	M.S.	E.M.S.	F
A	SS_1	$m - 1$	SS/DF	$\sigma^2_e + n\sigma^2_B + nr\sigma^2_A$	MSA/MSB
B	SS_2	$m(r - 1)$	SS/DF	$\sigma^2_e + n\sigma^2_B$	MSB/MSE
e	SS_3	$mr(n - 1)$	SS/DF	σ^2_e	
Totals	SS_T	$mrn - 1$			

Bennett and Franklin (1954) give the following steps for arriving at the EMS, leading to the proper test of significance, for experiments of any complexity:

---

* Refer to computational framework in any standard statistical text for value SS_1 through SS_T.

Factors are designated by capital letters (A, B, etc.). Levels are designated by small letters (*m*,*r*,*n*, etc.). Effects are designated by lower case letters relating to the respective Factors (a, b, etc.). A replication is indicated by parentheses, interactions as products (ab, etc.).

A table is prepared with Factors as column headings and Effects as row designations. Under each column heading is space for indicating the number of levels and the model, fixed or random.

The following rules are followed in filling out the table.

- In each column write opposite any row not containing the same letter as the heading, the number of levels.
- In each row containing an effect in parentheses, write 1 where letters are common to row and column.
- In remaining spaces, write 1, if the type is random; write 0, if the type is fixed.
- The EMS is obtained by multiplying in rows, all figures except those in columns having letters in common with the row, as illustrated.

Two-Factor Mixed Model, with Replication

Factor	A	B	E	
Level	m	r	n	
Type	R	F	R	EMS
a	1	r	n	$\sigma^2_e + rn\,\sigma^2_A$
b	m	0	n	$\sigma^2_e + n\,\sigma^2_{AB} + mn\sigma^2_B$
ab	1	0	n	$\sigma^2_e + n\,\sigma^2_{AB}$
e(ab)	1	1	1	σ^2_e

Effects a and ab are tested by the error term. Effect b is tested by the interaction term. The analysis of data from biological experiments is often complex because of non-linear variables, non-linear responses, high levels of variability, small sample sizes and other things that make careful application of statistics a necessity. The tests mentioned in this section are among the basic, most widely used ones. Each biological testing program has special requirements. For example, in bioassay of rats and mice for carcinogenicity of chemical substances, the following statistical methods are applied.

Survival probabilities are estimated by the product limit procedure of Kaplan and Meier (1958) and presented in the report in the form of graphs. Deaths due to accident or scheduled sacrifice are treated as censored observations and all other deaths are uncensored. Statistical tests of differences in survival between groups are made using the method of Cox (1972) for 2 groups and an extension of this method by Tarone (1975) for more than 2 groups.

The number of animals with tumors is analyzed as percentage of the number of animals pathologically examined. For some sites, such as liver or lung, the animal is entered in the denominator of the proportion of tumors at the site only if that site had a histologic examination. For tumors that may appear at several sites, any animal that had at least one such site histologically examined is entered in the denominator of the proportions given for that tumor.

Statistical analysis of tumor incidence is made using the Fisher exact test (Cox, 1970) to compare the controls to each dose level. In addition, the Armitage and Cochran test for linear trend in proportions with continuity correction (Armitage, 1971) is used. This test, assuming a linear trend, determines if the slope of the dose-response curve is different from zero ($P < 0.05$). The method also calculates the probability level of a departure from linear trend.

A conservative adjustment for simultaneous comparisons of several treatments with a control is the Bonferroni inequality (Miller, 1966). For the comparison of k doses with a control, this correction requires a significance level less than or equal to 0.05/k for the overall comparison to be significant at the 0.05 level. This adjustment is not made in the tables where the Fisher exact test results are shown but is discussed in the analysis when appropriate.

Other statistical methods are discussed in connection with specific biological experiments in later Sections.

2.3 SAMPLING

2.3.1 Background of Sampling

In U.S. EPA (1973a), Weber presents the concepts of sampling in biological research very concisely:

- An experimental unit is an object on which a measurement or observation may be made
- The set of all experimental units of interest in a study is the universe, or population
- A sample is a sub-set of experimental units, or of the measurements made on those units, usually only a small fraction of the population
- The sample must consist of a sufficient number of units (sample size) to represent the population, with the required precision and accuracy
- Sampling units or sampling points must be selected with known probability
- Random selection is necessary to satisfy the requirement for known probability
- A random sample, selected using a device such as a table of random numbers consciously has no bias

Experimental units may be discrete objects, such as test animals or, if interest is in spatial distribution or density of a population, or rate of change, may be units of space (volume, area, etc.). If the population is a bulk material, such as water, air, or feed, the sampling unit cannot be known until a sampling device is applied. Furthermore, it is necessary to take into account the dynamic nature of living populations. There are evident benefits to be gained from taking sampling considerations into account early in the planning stages of a study. The experimenter may often benefit from the advice of a statistician at this point.

For random sampling, it is necessary that each unit in the population have an equal probability of being selected. This means that the population must be identifiable.

2.3.2. Randomization Procedure

A simple randomization procedure is as follows (alternatively, random numbers might be generated by a computer program):

- Identify and number all the measurement units in the population. The total number of such units is N
- Determine the sample size, *n*
- From a random number table select numbers equal to the number of measurement units required for the sample. (See any mathematical or statistical textbook for the table.)
- Start at any random point in the table and read numbers consecutively in any direction
- Once a number has been selected ignore the recurrence in the table and read on until *n* numbers have been picked
- The correspondingly numbered units in the population constitute the sample

2.3.3 Sampling Models

Two models will probably satisfy most sampling requirements in biological research: simple (or unrestricted) random sampling (Model I), and stratified random sampling (Model II) (Bicking, 1976). Simple random sampling is used when the population is not subdivided. Stratified random sampling is used when the population is divided into strata or when a material is in divided or packaged form. Knowledge of the nature, content, and variability within strata is necessary in selecting the sampling scheme to be used. A pilot study may have to be made to obtain information about stratification. As a general rule, strata should be bounded in such a way that measurements are most alike within strata and most different between strata. In aquatic field situations, for example, stratification may be based on depth, bottom type, isotherms, or other variables (U.S. EPA, 1973a).

In field studies, a modified form of simple random sampling (systematic random sampling) may be desirable. A transect is laid out to be assured of including an adequate cross-section yet retaining ease of sampling. Placement of the transect should be at random. Also, a random starting point should be selected.

Randomness is used to reduce the possibility that large constant or systematic errors contribute to inaccuracy of the sample. Since accuracy also includes a component due to the variability of the measurement units within the sample, precision is also important.

2.3.4 Selection of Size of Sample

All the information necessary for the selection of a sample with the desired precision may not be available prior to sampling. As experience is acquired, even though there may have been very little information at first on the distribution of the property being measured in the population, sampling can be adjusted to meet precision requirements more exactly and more economically as information is acquired in early stages of the study. A valid estimate of precision can be made from the sample itself if it has been drawn according to an appropriate statistical probability model.

2.3.4.1 Sampling from a Normal Distribution Population--

If the population is homogeneous, a single sample unit may represent it adequately. However, even for water and other simple liquids (single phase liquids) it is possible that under certain conditions temporary stratification (caused by poor mixing or temperature gradients) may exist. This is the reason for arranging to get a composite sample by the act of sampling at several locations or several levels and compositing the subsamples thus obtained. This is always a good practice if the purpose is to obtain an average value for the property of the material.

If the population is not homogeneous, then a number of sample units should be drawn and analyzed separately, or composited and analyzed. If a prior estimate of the standard deviation is available, the sample size n

is calculated (using Model I) by:

$$n = (t\sigma'/D)^2$$

Eq. 2.3.1,

where σ' is the prior estimate of the standard deviation of the material, D is the maximum allowable difference between the estimate to be made from the sample and the actual value, and t is a probability factor to give a selected level of confidence that the difference is greater than D. See Bicking (1968).

Suppose that repeated sampling of a certain population had resulted in a standard deviation of 0.187 in measurements of the property of interest. The number of items required to assure with 95% confidence that the average quality of the population lies within the limits 0.15 of the average of the determinations is, from Eq. 2.3.1

$$n = (2 \times 0.187/0.15)^2 = 6.25 \text{ or } 7 \text{ items.}$$

When sufficient items have been tested to estimate the standard deviation from the data itself (say 30 as a minimum), sample size may be recalculated, if desired, using Eq. 2.2.5.

If the population is divided into distinct units or may be so divided in some suitable way or if it is stratified, and from these primary units (strata) secondary units (increments) may be taken by sampling, the most economic increment number and sample size are given by the following equation (using Model II):

$$k = \sigma'_w / \sigma'_b \sqrt{c_1 / c_2}$$

Eq. 2.3.2.

$$n = N(\sigma'^2_w + k\sigma'^2_b) \;/\; [Nk(D/t)^2 + \sigma'^2_b]$$

Eq. 2.3.3.

where σ'^2_w is the variance within secondary units averaged over all primary units; σ'^2_b is the variance between primary units; c_1 is the cost of preparing a primary unit; c_2 is the cost of taking a secondary unit; N is the number of primary units available for sampling; D is the allowable uncertainty in the sample result; and t is the probability factor. Equation 2.3.2 gives the number of secondary units per primary unit and Equation 2.3.3 the number of primary units in the sample.

The total cost of the sample can be represented by:

$$c = nc_1 + nkc_2$$

Eq. 2.3.4.

Accordingly, sample schedules can be set up for any set of conditions for which variances and cost can be determined, to make possible selection of samples with predetermined precision at minimum cost.

Consider a stream section having a series of ten pools. It is desired to determine the wet weight in mg of chironomid (midge) larvae in the bottom sediment of the stream. An Ekman dredge is to be used. In a previous experiment three dredge samples from each of four pools provided estimates of variance within pools equal to 0.84 mg and between pools equal to 2.35 mg (U.S. Geological Survey, 1973). The question is: how many pools should be sampled and how many dredge hauls be made per pool to determine the average chironomid weight per dredge haul within 1.0 mg? It was also known from previous experiments that the cost of moving the dredge and setting it up at a different pool costs 10 times as much as to collect a single sample where the dredge is already sited.

The number of dredge hauls per pool is calculated from Eq. 2.3.2 as:

$$k = \sqrt{0.84/2.35} \times \sqrt{10/1} = 1.89, \text{ or 2 hauls}$$

The number of pools to be sampled is (Eq. 2.3.3):

$$n = 10\,(0.84 + 2 \times 2.35)\ /\ [10 \times 2\,(1.0/2)^2 + 2 \times 2.35]$$
$$= 5.7, \text{ or 6 pools.}$$

Therefore, to minimize cost and the known error of sampling plan, two dredge hauls from each of six pools are required. The average weight in mg of these samples would be reported as the weight of chironomids per substrate area sampled per dredge haul.

2.3.4.2 Sampling from Non-normal Distributions--

For the bionomial distribution (example: proportion of occurrence in the population of an effect due to a treatment), sample size may be calculated, based on a prior estimate of presence of the effect, by Model I:

$$n = t^2\ \mathrm{PQ}/\mathrm{D}^2 \qquad \text{Eq. 2.3.5}$$

where P is the estimate of the presence of the effect, Q = (1 - P), and t and D are as in Eq. 2.3.1.

After the study is in progress, n can be recalculated, using the data itself, from Eq. 2.2.9.

2.3.5 Management of Sampling

The importance of sampling cannot be overlooked although there may be reasons why biological researchers have not always recognized probability based sampling as a necessary part of quality of results. A research laboratory is not like a service laboratory where the samples usually have been collected by someone from outside the laboratory and may even be blind samples for which the laboratory's main responsibility is analysis. Even in such circumstances, however, and much more so in a research laboratory, the validity of results is dependent not only on the precision and accuracy of tests and observations but also on the precision and accuracy of the sampling. Experimentation with improperly collected samples may well be wasted.

In the same sense that experiments should be designed, sampling should be designed. Looked at as a Model II design (i.e., a random factor design), the dependence of the final data output on the populations involved, the samples and the tests, is illustrated as follows:

Generalized Sampling Design

Population 1 . . . . m

Sample 1.1 .... 1.n m.1 .... $m.n$

Analysis 1.1.1 ... 1.1.r 1.n.1 ... 1.$n.r$ m.1.1 ... m.1.r $m.n$.1 ... $m.n.r$

The errors propogate throughout the system. Thus, the variance of the result is made up of components due to the test method, due to the sampling procedure and due to the non-homogeneity of the populations. It is the object of quality control to minimize these components or, where they cannot be made smaller, to balance the experiment so that their effects are felt to the same extent in all parts of the experiment.

In some biological research, samples are collected in the field either by the researcher or by a part of a team responsible to him. The necessity for good sampling practice begins in the field and extends to all aspects of the selection of test materials and test subjects, and even, in some instances, to selection of data.

The basic sampling models described in this section will require elaboration, particularly in field sampling. In the parts of the Guidelines dealing with specific areas of research, more details are given. The sampling sections of the biological testing methods given in Standard Methods (Rand et al., 1975) are very useful. Also, there are some very good recent EPA publications which should be referred to for sampling approaches in practice (U.S. EPA, 1973a, 1973b, 1974b, 1975).

Sampling usually presents a statistical problem, often substantial enough to require advice of a statistically trained person. The reason for this can be seen by reference to the basic formula for calculating sample size, $n = (ts/D)^2$. There must be information on the variance in measurements on similar samples (s^2), there must be a determination by the experimenter of the difference that is important to him (D), and a selection must be made of the probability level (determines size of t) at which decisions are to be made.

In some areas of biological research, particularly in new areas, or when new methods are being tried, very little information may be available on the variance of results. The experimenter must depend on experience and on theory to get early estimates of variances. One expedient is to err on the safe side and use very large sample sizes. This may be feasible in some areas, such as microbiological research where organisms are found in nature in very large accumulations or reproduce very rapidly. This way out becomes more difficult as the test subject becomes larger, or more expensive and the cost per test unit becomes larger. The point is that there are physical and economic limits on what can be done with increasing sample size. Where statistical theory is applicable, the sampling should be based on probability. Where background information consists of the scientist's input based on theory and experience, that should be used. Many sampling procedures designed without statistical help are very good because the scientist knows what he is dealing with. However, sampling should never be haphazard because then there is no control of errors or of costs and sampling may be overdone and wasteful or underdone and unreliable.

2.3.5.1 Chain of Custody--

One of the principal concerns in management of sampling is maintenance of systematic control of samples as they proceed from the field, through the laboratory tests, to disposition or storage. The control system is what is referred to as the Chain of Custody. Written records of the chain of custody are very important if results of sampling ever become evidence in litigation.

The chain of custody is very important in field sampling, when different organizations may be responsible for the sampling and the subsequent testing. It is also very important when it is necessary to maintain parts of the original samples as reference samples for future checking or for independent investigators. It is equally necessary that good procedures be used in biological research where samples of various kinds are important.

Test substances should be carefully controlled because identity, stability, inventory control, integrity of the sample and safety are important.

Test subjects may be obtained from supply laboratories or may be bred or cultured within the laboratory. Identity of individual subjects, the record of treatments, observations on individuals and groups, remains of sacrificed or dead subjects, all need to be controlled by a good system.

Keeping in mind that in a biological research laboratory the samples may be chemicals, organisms in treatment groups, samples of organisms or parts thereof and organs, tissues, etc. for clinical tests or histopathology, the problem becomes a general one of responsibility, record keeping, secure storage, and all other activities necessary to maintain integrity of results.

Some of the important aspects of a chain of custody system for biological research are:

- Clear assignment of responsibility of keeping track of samples of all kinds at all program stages
- Designation of secure storage space for all research materials when not in actual course of experimentation
- Handling of samples by a minimum number of persons
- When samples are transferred, receipt or dispatch should be handled by one person who keeps a complete record of all transactions
- All samples should be appropriately identified and the identification should be recorded in a permanent log book
- While in the course of experimentation all samples should be in possession or view of the experimenter or

appropriately secured

- The record should include accounting for unused portions of samples and disposition of samples when a program is completed
- All residual materials and records should be retained until an agreed-upon retention period expires

The Chain of Custody record is an important part of the complete record system.

2.3.5.2 Sample Preservation and Handling--

For the water environment, recommendations for preservation and holding of samples are given in Table 2.2 (EPA, 1974b). The holding time given in the table is interpreted as the recommended maximum period between sampling and anaylsis. Preservatives, where specified, are required to ensure stability for the holding time. If holding times are exceeded, a notation of that fact should be made on data sheets before they are transmitted.

For some tests, to exceed the maximum holding time would very seriously compromise the accuracy of the measurement. The parameters to which this applies include the following:

Biochemical Oxygen Demand
Cyanide, Total
Chlorine, Total Residual
Phenols
Turbidity
Streptococci Bacteria
Coliform Bacteria
Temperature

Microbiological sampling requirements are to be found in Section 405 of "Standard Methods" (Rand et al., 1975) and radiological sampling requirements in Sections 200 and 300A of the same reference.

For biological organisms, the pertinent information will be found in Section 3.1.4 and the other sub-sections of Section 3 dealing with specific biological areas.

TABLE 2.2 RECOMMENDATIONS FOR SAMPLING AND PRESERVATION OF WATER SAMPLES ACCORDING TO MEASUREMENT(1) (U.S. EPA, 1974b)

Measurement	Volume Required (ml)	Type of Container	Method of Preservation	Holding Time (6)
Acidity	100	P, G(2)	Cool, 4°C	24 hours
Alkalinity	100	P, G	Cool, 4°C	24 hours
Arsenic	100	P, G	HNO_3 to pH<2	6 months
BOD	1000	P, G	Cool, 4°C	6 hours (3)
Bromide	100	P, G	Cool, 4°C	24 hours
COD	50	P, G	H_2SO_4 to pH<2	7 days
Chloride	50	P, G	None required	7 days
Chlorine req.	50	P, G	Det. on site	No holding
Color	50	P, G	Cool, 4°C	24 hours
Cyanides	500	P, G	Cool, 4°C NaOH to pH 12	24 hours
Dissolved oxygen				
Probe	300	G only	Det. on site	No holding
Winkler	300	G only	Fix on site	4 to 8 hours
Flouride	300	P, G	Cool, 4°C	7 days
Hardness	100	P, G	Cool, 4°C HNO_3 to pH<2	7 days
Iodine	100	P, G	Cool, 4°C	24 hours
MBAS	250	P, G	Cool, 4°C	24 hours
Metals				
Dissolved	200	P, G	Filter on site HNO_3 to pH<2	6 months
Suspended			Filter on site	6 months
Total	100		HNO_3 to pH<2	6 months

(continued)

TABLE 2.2 (Continued)

Measurement	Volume Required (ml)	Type of Container	Method of Preservation	Holding Time (6)
Mercury				
Dissolved	100	P, G	Filter HNO_3 to pH<2	38 days (Glass) 13 days (Hard plastic)
Total	100	P, G	HNO_3 to pH<2	38 days (Glass) 13 days (Hard plastic)
Nitrogen				
Ammonia	400	P, G	Cool, 4°C H_2SO_4 to pH<2	24 hours(4)
Kjeldahl (total)	500	P, G	Cool, 4°C H_2SO_4 to pH<2	7 days (4)
Nitrate	100	P, G	Cool, 4°C H_2SO_4 to pH<2	24 hours(4)
Nitrate	50	P, G	Cool, 4°C	24 hours(4)
NTA	50	P, G	Cool, 4°C	24 hours
Oil & grease	1000	G only	Cool, 4°C H_2SO_4 to pH<2	24 hours
Organic carbon	25	P, G	Cool, 4°C H_2SO_4 to pH<2	24 hours
pH	25	P, G	Cool, 4°C Det. on site	6 hours(3)
Phenolics	500	G only	Cool, 4°C H3PO4 to pH<4 1.0 g. $CuSO_4$/1	24 hours

(continued)

TABLE 2.2 (Continued)

Measurement	Volume Required (ml)	Type of Container	Method of Preservation	Holding Time (6)
Phosphorus				
Orthophosphate Dissolved	50	P, G	Filter on site Cool, 4°C	24 hours(4)
Hydrolyzable	50	P, G	Cool, 4°C H_2SO_4 to pH<2	24 hours(4)
Total	50	P, G	Cool, 4°C	7 days (4)
Total Dissolved	50	P, G	Filter on site Cool, 4°C	24 hours(4)
Residue				
Filterable	100	P, G	Cool, 4°C	7 days
Non-filterable	100	P, G	Cool, 4°C	7 days
Total	100	P, G	Cool, 4°C	7 days
Volatile	100	P, G	Cool, 4°C	7 days
Settleable Matter	1000	P, G	None required	24 hours
Selenium	50	P, G	HNO_3 to pH<2	6 months
Silica	50	P only	Cool, 4°C	7 days
Specific Conductance	100	P, G	Cool, 4°C	24 hours(5)
Sulfate	50	P, G	Cool, 4°C	7 days
Sulfide	500	P, G	2 ml zinc acetate	24 hours
Sulfite	50	P, G	Det. on site	No holding
Temperature	1000	P, G	Det. on site	No holding

(continued)

TABLE 2.2 (Continued)

Measurement	Volume Required (ml)	Type of Containter	Method of Preservation	Holding Time (6)
Threshold odor	200	G only	Cool, 4°C	24 hours
Turbidity	100	P, G	Cool, 4°C	7 days

(1) More specific instructions for preservation and sampling are found with each procedure as detailed in this manual. A general discussion on sampling water and industrial wastewater may be found in ASTM, Part 24, p. 72-91 (1973)

(2) Plastic or glass

(3) If samples cannot be returned to the laboratory in less that 6 hours and holding time exceeds this limit, the final reported data should indicate the actual holding time

(4) Mercuric chloride may be used as an alternate preservative at a concentration of 40 mg/l, especially if a longer holding time is required. However, the use of mercuric chloride is discouraged whenever possible

(5) If the sample is stabilized by cooling, it should be warmed to 25°C for reading, or temperature correction made and results reported at 25°C

(6) It has been shown that samples properly preserved may be held for extended periods beyond the recommended holding time

2.4 PRECISION AND ACCURACY OF TESTS

2.4.1 Measurement of Precision and Accuracy

A laboratory must have a well-organized and clearly defined program to check the validity of the data it produces. Validity is usually expressed in terms of precision and accuracy. Precision is the reproducibility among replicate observations and accuracy is the difference between observed and known, or actual, values.

An analyst initially may establish the precision of a particular method by a minimum of 5-10, preferably 30, replicate determinations on a single sample. Generally, it will be necessary to repeat this procedure on each type of sample that will be analyzed by a given method and preferably on several samples of each type from each source. Comparison of the precision obtained with reference standards and that obtained with actual samples will reveal any interferences from contaminants in the samples.

The standard deviation of the individual measurements is the basic number for expressing precision. The smaller the standard deviation, the better the precision. There are various ways in which the standard deviation may be used in presenting precision. One of the most widely accepted ways is to use precision limits:

$$P = \pm ts$$

where t is a probability factor (approximately equal to 2.0 for 95 percent limits of precision) and s is the calculated standard deviation. The ASTM Standard for expressing precision (ASTM, 1977) gives other ways of presenting precision.

It may be desired to determine the precision of an average. Then, precision of the average is

$$P_{\bar{x}} = \pm\ ts\ /\ \sqrt{n}$$

where n is the number of measurements in the average.

The accuracy of a method may be determined initially by a minimum of 5-10, preferably 30, replicate analyses of samples to which known amounts of reference standards have been added (spiked samples). The results should be reported as percent recovery at the final concentration of the spiked sample. The spiking of actual samples for these determinations allows for a more realistic measurement of accuracy than the exclusive use of pure reference standards, although again comparison of the accuracy obtained with spiked samples and that obtained with reference standards may be of interest in identifying sources of error. Analysis of blanks also will be important for many paramenters where background level may be non-zero and where a blank correction may be necessary.

It should be noted that there is some uncertainty (imprecision) in the calculation of percent recovery. The precision of the average percent recovery may be calculated as above. Strictly speaking, the percent recovery measures the bias in the method, and accuracy should be expressed as the bias plus or minus the precision of the average percent recovery.

2.4.2 Control of Precision and Accuracy

2.4.2.1 Use of Standard Methods--

The availability of standard test methods is one of the indicators of maturity of a scientific discipline. In industry and in regulatory activities the need for standard methods is obvious to assure comparability of results and as a basis for adjudication. In scientific research, the requirement of flexibility has been used as a justification for caution in the development of rigid standards. In new disciplines, the development of the test methods is a part of the research problem. However, the extent to which attention is given to standards development is a measure of the trustworthiness of the major scientific results.

In biological research, the experimental protocol may itself be the test, with the animal subject serving as the instrument. If this view is accepted, there can be no excuse for delay in moving toward standard protocols. The requirement of good science, that results can be verified by other investigators and at other times and places, is a sufficient imperative.

It is sometimes suggested that standardization and other quality control activities are appropriate only where routine, meaning repetitive, measurements are made. Such an argument can be made logically only when the research is truly basic. A novel method of test may be the key to successful research. Even the keenest researcher may not be able to write the rules in advance. But biological research to which society has committed itself has moved the experimenter out of the ivory tower and there can be no valid pretense that the science is not applied science. The increased availability of standard methods of test is a requirement for progress.

In Section 2.6 there is given a Guide to the Preparation of Specifications and Standards, which suggests, among other things, a format for standard methods of test. An example is given of a standard method of test for purity of chemicals for use in a bioassay program. In the various parts of Section 3, covering different kinds of bioassay, sample bioassay protocols are given.

Copies of all methods in use should be collected, preferably in a loose-leaf binder, and kept in a place readily accessible to the researcher. Performance should be closely supervised to assure that all testing is by approved, standard methods.

2.4.2.2 Maintenance and Calibration of Instruments--

Maintenance and calibration of instruments are critical to the generation of good data. Instruments and apparatus must be maintained in good working order, calibrations must be performed in an appropriate manner and with sufficient frequency, and records and documentation of maintenance and calibration must be adequate.

Someone in the laboratory should have the responsibility to see that each of the instruments is properly maintained and calibrated on schedule. This may or may not be the same person who actually does the maintenance and calibration. The important thing is that the responsibility be clearly assigned.

For legal and scientific reasons, it is important to keep careful records of maintenance and calibration of instruments and apparatus. Generally, these records should be kept in permanent (bound) notebooks in ink with each entry signed and dated. A separate log (or a separate section of a log) should be assigned to each instrument or piece of apparatus that requires any sort of periodic calibration or maintenance, whether that activity is performed by laboratory personnel or by an outside agency under contract. It is convenient to include all calibration, maintenance, and repair actions on an instrument in the log, as a complete and accessible record of the condition of that instrument. This includes traceability of standards to the National Bureau of Standards or other recognized source.

Each entry must specify clearly what action was taken when and by whom. For example, if a new calibration curve was established which will be the basis for future analyses, either the curve or a reference to a notebook containing the curve should be included, along with an explanation of how the curve was established (identification of reference standards, methodology) and when the analyst began using the curve.

The critical factors are the calibration and maintenance procedures and the frequency and regularity with which they are carried out. This information should appear in the instrument calibration and maintenance logs and the laboratory quality control manual.

Calibration recommendations for some of the major instruments are included in Table 2.3. These recommendations are not to be considered as rigid rules but rather as guidelines in controlling laboratory performance. It is recognized that optimum procedures may vary somewhat as a function of instrument manufacturer and model. Additional materials that could be useful to the scientist are operation and maintenance manuals for the various instruments.

2.4.2.3 Routine Control of Test Performance--

After the precision and accuracy of the method are established, the analyst will need to incorporate replicates, spikes, standards, and blanks, as appropriate, into the sequence of routine analyses to insure that valid data are being generated. The frequency and procedures required for adequate monitoring of the quality of the data will depend on the method itself. The experience of conscientious analysts and statisticians in the field is an invaluable source in this matter. For example, one group of chemists experienced on the Technicon Auto Analyzer usually runs a duplicate, a spiked sample, and a reference standard every 8 samples in a large series of similar samples, or one in each set of samples, whichever is more frequent. A chemist experienced in the analysis of phenols and cyanide suggests verifying the standard curves each day, that these parameters are analyzed with a low and a high reference standard and a blank, and running a duplicate and a spike with each small set of samples. Gas chromatography often requires multiple injections of the sample with and without an internal standard, in addition to spiked samples and a blank, for each sample analyzed. These examples are given only to demonstrate how quality control protocols will vary considerably with the method and the experience of the analyst. The nature of the samples (simple or complex mixtures), the condition of the instrument, the importance of the sample, the breadth of the precision and accuracy control limits, and many other factors may also affect the quality control requirements.

Because there are no universal guidelines for the frequency and procedures required in the use of quality control samples, it is very important that each laboratory develop its own internal guidelines based on sound statistical methods and experience. These should be in the form of written, explicit protocols for each parameter or group of parameters. Some techniques for quality control of instruments are outlined in Table 2.4.

It is of primary importance that the analyst and the laboratory have a proper appreciation of the importance of replicates, spikes, standards, and blanks in assuring the validity of their analytical data.

It should be noted that a popular method of monitoring daily performance has been the use of Quality Control Charts. Basically, these charts, constructed separately for each method or parameter, display the control limits for precision and accuracy, and the actual precision and accuracy measured from day to day, and provide a continuous visual picture of the control of data quality for that method or parameter. Details of control chart construction will be found in Section 2.10.

TABLE 2.3 INSTRUMENT CALIBRATION (U.S. EPA, 1978)

Instrument	Procedure	Frequency
1) Analytical balances	(a) Zero (b) Standard weights (c) Full calibration and adjustment	(a) Before each weighing (b) Monthly (c) Annually
2) pH meters	At pH 4, 7, and 10	Daily
3) Conductivity meters	(a) Obtain cell constant with potassium chloride reference solutions	Daily
	(b) Construct temperature curve if measurements are to be made other than at 25 ± 0.5°C	Monthly
4) Nephelometer/ turbidimeters	(a) Check instrument scales or develop calibration curve with formazine stds	Monthly (≤40NTU)

TABLE 2.3 (continued)

Instrument	Procedure	Frequency
	(b) If manufacturer's stds. are not formazine, check against formazine stds. (≤40NTU)	Annually
5) Colorimeters/filter photometers	Curves determined with 5 to 6 laboratory-prepared std. solutions for each parameter in conc. range of samples	Daily
6) UV/visible	(a) Wavelength calibration with holmium oxide glass or solution, low-pressure mercury arc, benzene vapor (UV), or hydrogen arc (visible)	Quarterly
	(b) Absorbance vs. concentration curves with 5 to 6 std. solutions for each parameter at analytical wavelength in conc. range of samples	Daily
	(c) Full servicing and adjustment	Annually
7) Infrared spectrophotometers	(a) Wavelength calibration with polystyrene or indene	Daily
	(b) Absorbance vs. concentration curves with 5 to 6 std. solutions for each parameter at analytical wavelength in conc. range of samples	Daily
	(c) Full servicing and adjustment	Semi-Annually
8) Atomic absorption spectrophotometers	(a) Response vs. concentration curves with 6 to 8 std. solutions for each metal (std. mixtures are acceptable, but with same acid as sample to be run) in conc. range of samples	Daily
	(b) Full servicing and adjustment	Annually
9) Carbon analyzers	Curves determined with 5 to 6 std. solutions in conc. range of samples	Daily

(continued)

TABLE 2.3 (continued)

Instrument	Procedure	Frequency
10) DO meters	Calibrated against modified Winkler method on aerated distilled or tap water	Daily
11) Other selective ion electrodes and electrometers	Curves determined with 5 to 6 std. solutions in conc. range of samples	Daily
12) Thermometers	Calibrate in constant temperature baths at two temperatures against precision thermometers certified by NBS	Quarterly
13) Technicon auto analyzers	(a) Curves determined with std. solutions for each parameter	Each set of samples
	(b) Full service and adjustment (esp. colorimeter)	Annually
14) Gas chromatographs	(a) Retention times and detector response checked with std. solutions	Daily
	(b) Response curves for each parameter determined with std. solutions	Monthly
15) Radiological equipment	(See Standard Methods, Sect. 300)	
16) Sulfur dioxide in air sampler/analyzers (pararosaniline method)	(a) Calibrate flowmeters and hypodermic needles against a wet test meter	Quarterly
	(b) Spectrophotometric calibration curve with 5 to 6 std. sulfite-TCM solutions at controlled temperature ($\pm 1°C$)	Monthly
	(c) Sampling calibration curve with 5 to 6 std. atmospheres from permeation tubes or cylinders	Monthly
	(d) Calibrate associated thermometers, barometers, and spectrophotometer (wavelength)	Quarterly
17) Suspended particulates (high-volume sampler method)	(a) Calibrate sampler (curve of true air flow rate vs. rotameter or recorder reading) with orifice calibration unit and differential manometer at 6 air flow rates	Monthly

(continued)

TABLE 2.3 (continued)

Instrument	Procedure	Frequency
	(b) Calibrate orifice calibration unit with positive displacement primary standard and differential manometers	Annually
	(c) Calibrate relative humidity indicator in the conditioning environment against wet-bulb/dry-bulb psychrometer	Semi-annually
	(d) Check elapsed time indicator	Semi-annually
	(e) Calibrate associated analytical balances, thermometers, barometers	As needed
18) Carbon monoxide (non-dispersive IR)	(a) Determine linearity of detector response (calibration curve) with calibration gases (0, 10, 20, 40, and 80% of full scale, certified to ±2% and checked against auditing gases certified to ±1%)	Monthly
	(b) Perform zero and span calibrations	Before and after each sampling period
	(c) Calibrate rotameter and sample cell pressure gauge	Semi-annually
19) Photochemical oxidants (ozone)	(a) Calibrate standard KI/I_2 solutions in terms of calculated O_3 equivalents at 352 nm	Weekly
	(b) Calibrate instrument response with 6 to 8 test atmospheres from ozone generator, spanning expected range of sample concentrations (usually 0.05-0.5 ppm O_3)	Monthly
	(c) Calibrate flowmeters, barometer, thermometer	Semi-annually
	(d) Calibrate and service spectrophotometer	As specified

(continued)

TABLE 2.3 (continued)

Instrument	Procedure	Frequency
20) Hydrocarbons (corrected for methane)	(a) Determine linearity of detector response with calibration gases (0, 10, 20, 40, and 80% of scale, certified to ±2%)	Monthly
	(b) Perform zero and span calibrations	Before and after each sampling period
	(c) Calibrate flowmeters and other associated apparatus	Semi-annually
21) Nitrogen dioxide (arsenite 24-hr. sampling method)	(a) Calibrate flowmeter with wet test meter	Monthly
	(b) Calibrate hypodermic needle (flow restrictor) with flowmeter	Each new needle
	(c) Obtain colorimetric calibration curves with 5 to 6 std. nitrite solutions	Weekly
22) Nitrogen dioxide (Griess-Saltzman colorimetric, continuous)	(a) Dynamic calibration with std. atmospheres (e.g., from permeation tubes) spanning the range of observed concentrations	Monthly
	(b) Static colorimetric calibration with 5 to 6 std. nitrite solutions	Weekly
23) Nitrogen dioxide (chemiluminescence, continuous)	(a) Calibrate std. NO cylinder with ozone generator (precalibrated by iodometric procedure)	Each new cylinder
	(b) Calibrate NO monitor with std. NO cylinder at several concentrations	Monthly
	(c) Calibrate NO_2 monitors with std. NO cylinder (diluted NO concentrations determined with NO monitor) and calibrated ozone generator	Monthly
	(d) Calibrate associated flowmeters	Semi-Annually

(continued)

TABLE 2.3 (Continued)

Instrument	Procedure	Frequency
24) Autoclaves and sterilizers	(a) Sterilization effectiveness checked (e.g., _B._ _stearothermophilus_, color indicator tape for ethylene oxide)	Daily
	(b) Temperature-recording device calibrated	Semi-annually

TABLE 2.4 TECHNIQUES FOR QUALITY CONTROL OF INSTRUMENTS (ASTM, 1977)

Control Parameter	Control Technique
Instrument operating range	Coordinate instrument selection with method requirements
Interferences	Sample conditioning (drying, separating, mixing, etc.)
	Use of blanks
	Use of spiked samples
Environmental conditions	Monitor and control temperature, humidity, pressure, and atmospheric parameters that can affect system response. Consult manufacturer's instructions and method descriptions.
Associated equipment operation (cuvettes, volumetric ware, dilutors, etc.)	Proper handling procedures
	Standard procedures for cleaning
	Standardization or calibration
Normal system drift	Zero adjust
System component functions	Apply function tests
	Plot response to changing concentrations
	Perform maintenance when indicated
Response readout	Use calibration curve, adjust using blanks and zero-span controls

2.5 PHYSICAL ENVIRONMENT OF RESEARCH

The environmental factors in the research laboratory can affect the quality of sampling and observation. Good housekeeping provides the proper setting for a quality control program. Some effects of poor housekeeping are related to occupational safety and health, which are important. Lack of care also usually goes with poor maintenance which leads to deterioration in the quality of data. Some elements of poor housekeeping practices, which quality-minded management will guard against are given in Table 2.5 (U.S. EPA, 1973b).

Laboratory support services require quality control. Services include gases, water, electricity and space conditioning. Some of the parameters of support services that affect quality, and suggested control techniques are given in Table 2.6 (U.S. EPA, 1973b).

Purchasing guides, or specifications, are required for all expendable materials used by the laboratory. Purchasing and acceptance specifications are discussed in Sect. 2.6. The same considerations apply to purchased support services.

The quality of reagent water is a matter deserving special attention. If the water has been purchased, each batch should be tested for conductivity before acceptance. High purity water is generally defined as water having a conductivity of 2.0 micromhos or greater. It may be necessary to redistill water if greater purity is required. Stills, storage tanks and piping must be specified, installed and maintained so as to minimize contamination. Pretreatment of feed water will improve still operation. Ion exchange resins are used to remove calcium and magnesium. A carbon filter on the feed water intake will remove organic materials. Certain needs in biological research may call for double- or triple-distilled water.

Also it may be a requirement that the water be ammonia-free, carbon-dioxide-free, or ion-free. Ion exchange columns using research grade cartridges can produce high quality water (ASTM Referee Reagent Grade) with a maximum of 0.1 mg/l total matter and maximum conductivity of 0.1 micromho.

TABLE 2.5 EFFECTS OF HOUSEKEEPING PRACTICES ON LABORATORY PERFORMANCE (U.S EPA, 1973b)

Element	Possible Effects
Excess atmospheric or accumulated dust	Failure of electrical contacts and switches, excessive wear of mechanical components, excessive soiling of optical components
Reagent spillage or leaks	Corrosion, hazardous vapors, electrical hazards, insecure footing
Improper maintenance of air conditioning and heating equipment	Air conditioning and heating equipment failure, operation outside of designated limits, equipment damage, freezing, inking pen failures, excessive reagent evaporation
Improper use of extension cords or overloading of circuits	Poor voltage control, excessive circuit failures, electrical hazard
Improper cleaning of glassware and reagent containers	Reagent contamination
Non-systematized storage of parts and tools	Loss of tools, absence of tools and parts when required, subsequent system failure

TABLE 2.6 TECHNIQUES FOR QUALITY CONTROL OF LABORATORY SUPPORT SERVICES (U.S. EPA, 1973b)

Support Service	Parameters Affecting Quality	Control Techniques
Laboratory gases	Purity specifications - vary among manufacturers	Develop purchasing guides
	Variation between lots	Overlap use of old and new cylinders
	Atmospheric interferences	Adopt filtering and drying procedures
Reagent water	Commercial source variation	Develop purchasing guides - Batch test for conductivity
	Purity requirements	Redistillation, heating, deionization with ion exchange columns
	Atmospheric interferences	Filtration of exchange air
	Generation and storage equipment	Maintenance schedules from manufacturer recommendations
Electrical service	Voltage fluctuations	Battery power
		Constant voltage transformers
		Separate lines
		Motor generator sets
Ambient conditions	Temperature	Heating and air conditioning systems
	Humidity	Humidity controls

2.6 CHEMICALS AND REAGENTS

The quality control plan should include standard procedures for choosing chemicals, preparing standard solutions, storing and handling chemicals and reagents, and choosing and handling standard reference materials. Table 2.7 (U.S.EPA,1973b) lists some of the factors affecting such procedures with some of the appropriate control techniques.

2.6.1 Purchase Specifications

Chemical reagents, solvents and gases are available in a range of purities from technical grade to ultrapure grades. For many purposes, analytical reagent grade or pesticide grade will be satisfactory. Other uses, such as trace analysis or treatment in biological assay, will require special grades of purity. If purity is not specified, it is generally understood that analytical reagent grade is wanted. However, the chemical procurement specification should always state the desired chemical and physical properties and the purity required.

For most grades, it will be sufficient to specify grade based on the manufacturer's published data sheets, and acceptance may be on the basis of the supplier's certification without sampling and testing. Pure grades may have to be specified in detail and, depending on criticality of use, may have to be sampled and tested before dilution and use.

At this point, it is pertinent to consider the whole matter of the preparation of specifications and standards. Specifications and standards are required not only for chemicals and reagents but also for purchase of facilities, equipment, and supplies of all kinds; for field and laboratory operating procedures; for methods of test, including bioassay protocols; and for quality assurance procedures. The next sub-section gives a guide for specification and standard preparation in general.

2.6.1.1 Guide to the Preparation of Specifications and Standards --

- Introduction

This "Guide" provides the basis for the preparation of a system of specifications and standards in conformance with regulatory requirements and with current good laboratory practices. It provides the framework for a system suitable to health effects research, biological research, and environmental research in general.

- General Philosophy of Specifications and Standards
    - Definitions

A specification is a precise statement, usually for use in procurement, of the requirements for a material, product, system or service, including the procedure by which it can be determined that the requirements have been met within the limits specified in the statement.

A standard is a document containing a set of conditions to be fulfilled by an item, process or method based on the consolidated results of science, technique and experience which is approved by a recognized authority and usually determined to be acceptable to all to whom it may apply.

- Basic Considerations

The definitions of specification and standard agree with those approved by the International Standardization Organization, and are in accordance with the Federal Standards. A specification may be a standard, a part of a standard, or independent of a standard.

It is understood that the complete specification system shall have been committed to writing.

The purpose of specifications and standards in a research program is to ensure the validity and integrity of the data produced. Validity refers to the scientific faultlessness of the data and integrity refers to its presentation in unaltered form. The quality of results depends on appropriate control and verification procedures in the respective parts of the system.

- Categories of Documents

Materials specifications (or purchase specifications)

Standard Operating Procedures (Good Laboratory Practices)

Standard Bioassay Protocols

Standard Methods of Test, including Histology and Pathology

Quality Assurance Procedures

- Suggestions for the Preparation of Specifications and Standards

Requirements, as far as practicable, should be expressed in numerical terms and must include acceptable levels or limits of permissible variation.

The language used should contain the simplest words and phrases that will convey the intended meaning. Use "shall" whenever a specification expresses a provision that is binding; use "should" or "may" to express non-mandatory provisions. "Will" may be used to express a declaration of purpose on the part of the Government or where simple futurity is to be expressed.

Measurements shall be expressed in units of the metric system in accordance with the International System of Units (SI) as detailed in the National Bureau of Standards Special Publication 330. Equivalent units may be given in parentheses.

- Recommended Coding of Specifications

As a means of identification of specifications a uniform code system combining letters and numerals is established.

The first two letters of the Code are reserved to indicate the particular program. The next letter will indicate the category of specification as follows:

Materials Specification	M
Operating Procedure	O
Bioassay Protocol	P
Method of Test	T
Quality Assurance Procedure	Q

Succeeding numerals will identify the particular specification uniquely.

Example: CBP1 could indicate the carcinogenesis bioassay protocol for an Acute Toxicity Test.

- Recommended Format of Specifications

  o General outline form should be used. Each section should be numbered in arabic numerals and subsections in decimal notation. Active voice should be used. Tables may be used, for convenience, except that the clarity and completeness of the written specification shall not be sacrificed for brevity.

  o Information Common to Headings of All Specifications

Specification Number

Type of Specification

Page Number

Title

Approval. Initials of the person authorized to approve for each organizational unit should appear.

  o Content of Specifications

The following section headings shall be included in all documents in the system, with the note "Not Applicable," if the section is not required.

1. Scope
2. Applicable Documents
3. Requirements
4. Quality Control
5. Packaging (Materials specifications only)
6. Notes
7. Reference Documents
8. Appendix

Scope. A clear, concise delineation of the extent or range of technical content shall be given which may be clarified as needed by naming specific exclusions from coverage. A subparagraph headed "Application" may be included to indicate the general field or particular area of use.

Applicable Documents. Government or nongovernment specifications and standards may be referenced. Government regulations or codes may also be referenced if essential. Only documents identified in Sections 3, 4 and 5 of the specification that are supportive to or clarifying requirements of those sections shall be listed in Section 2. Referenced documents shall be currently available.

Requirements. All necessary requirements (materials, processes, systems and performance characteristics) shall be given. Only those characteristics should be stated that can be confirmed by reliable quality criteria or test equipment.

Quality Control. This section shall describe all sampling, testing and analyses to be performed to control specified procedures and supervisory actions to assure that the results conform to the requirements.

Packaging. Packaging is defined as the means of providing protection to items during shipment, storage, or redistribution operations.

Notes. This section shall contain information of a general or explanatory nature.

Reference Documents. Information sources are located in this section.

Appendix. Large data tables or detailed procedures or management plans may be appended to the specification. Such material applies to references in the body of the specification.

- Control of Specifications
    - Preparation and Distribution

This specification system applies to the whole of a particular program, and its staff is responsible for the identification of existing specifications and for obtaining or preparing new specifications. The program will maintain a complete file of specifications and subcontractors will maintain files of all specifications applicable to them.

- Review and Approval of Specifications

Approvals are required of the Program Director. Specifications must be accepted by sub-contractor to which they apply. The Program's Quality Control Officer shall review specifications based on the following criteria:

Conformance to coding, categories, and format

Provision of acceptable limits of variability

Inclusion or reference to a procedure for verifying that specification limits have been met

Necessary approvals and acceptances.

Specifications for a Mammalian Bioassay Program include

- Standard Bioassay Protocol
- Physical Plant and Material Specifications
- Good Animal Care Laboratory Practices
- Standard Methods of Test
- Safety Standards

Examples of specifications prepared in accordance with the Guide are given in the following pages: a physical plant specification, a materials specification and a standard method of test.

Examples of bioassay protocols in a format which departs considerably from the format suggested in the Guide will be found in the parts of Section 3 for specific kinds of bioassay. See Sect. 2.7.2 and Appendix B for a complete set of Good Animal Care Laboratory Practices suitable for mammalian bioassay with rodents.

TABLE 2.7 GUIDELINES FOR QUALITY CONTROL OF CHEMICALS AND REAGENTS (U.S.EPA, 1973b)

Procedure	Control Parameter	Control Technique
Choice of chemicals	Manufacturer designations	Develop purchasing guides
	Method purity specifications	Use American Chemical Society designations as a base
		Develop purification or treatment procedures specified by method
Preparation of standard solutions	Calibrated glassware	Purchasing guidelines
	Standard reference materials (SRM)	Schedules for restandardization of solutions
	Stability	
Storage and handling	Container composition	Design a labeling system
	Filtering or pretreatment	Purchase single lot numbers
	Environmental sensitivity	Rotate stock
		Control temperature, light, atmospheric exposure
Standard reference materials	Availability	Store in temperature-controlled atmosphere
	Stability	Desiccate when necessary
		Replace if instability is suspected
		Weigh to determine loss or degradation

Carcinogen Bioassay Program

Specification No. CBM-2

Type: PHYSICAL PLANT SPECIFICATION	**Sheet** 1	**Of** 6

Subject: BARRIER FOR PREVENTION OF CONTAMINATION BY PATHOGENIC MICROORGANISMS

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers considerations for the location and construction materials of the barrier system. Ideas on the room size and floor plan of the barrier system are also mentioned. Equipment areas, laboratories and quarantine area within the barrier system are described. A list of ancillary equipment for the barrier system is given. Lastly, the four different types of barrier system are suggested.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Location

3.1.1 Preferably, the barrier should be remote from other buildings or activities that could endanger its operation.

3.1.2 If it is part of a building, there should be a maximum isolation. This could be achieved by:

3.1.2.1 Spearate heating systems.

3.1.2.2 Installation of devices to prevent backflow through drains.

3.1.2.3 Containment of water leaks.

3.1.2.4 Use of differential air pressure to control air flow.

3.1.2.5 Separate access and egress corridors.

3.1.2.6 Controlled access by personnel.

3.2 Construction Materials

3.2.1 Interior materials should be chosen for durability, longevity, and low maintenance.

3.2.2 They should be resistant to corrosion, scrubbing, and harsh chemicals, but they should be easy to clean.

3.2.3 The entire facility must be protected from climatic conditions, and the building must be secure against such organisms as insects, wild rodents, and vermin.

3.3 Room size.

3.3.1 It is easier to contain a point outbreak of disease if the animal rooms are small and independent from each other.

3.3.2 Rooms should not contain more than one animal species.

3.3.3 Ideally, rooms should not be so large as to contain more cages than can be serviced by one person.

3.4 Floor plan.

3.4.1 The relation of one room to another and one floor to another will be dictated by the functions (in addition to animal care) of the facility and by the flow of people, supplies, animals, and so on through the facility.

3.4.2 The traffic pattern should avoid backflow from any area to a cleaner area.

3.4.3 The animal rooms are to be the most protected area.

3.5 Equipment areas.

3.5.1 All mechanical equipment should be located where it can be serviced without having the service personnel enter the more protected areas of the barrier.

3.5.2 Piping of any kind should not run directly over animal rooms but should be located above corridors.

3.6 Laboratories.

3.6.1 Areas outside of the animal rooms where animals will be handled must also be designed for ease of cleaning and have features to minimize possible contamination of animals by handling procedures.

3.6.2 Animals removed to conventional laboratories outside the barrier should not be brought back into animal rooms.

3.6.3 Consideration should be given to providing clean laboratories within the barrier with direct access to animal rooms.

3.7 Quarantine Area.

3.7.1 If animals are brought in from an outside source, or if animals removed from the barrier are to be returned, a protected area must be provided where they can be held until their freedom from contaminants is determined.

3.8 Ancillary Equipment.

3.8.1 The selection of the ancillary equipment, its placement, performance monitoring, servicing, and dependability play a major role in the success or failure of a barrier system.

3.8.2 Major movable and nonmovable equipment may be divided into the following categories:

3.8.2.1 HVAC (heating, ventilating, and air conditioning): Air-handling equipment, refrigeration compression or steam absorption equipment, humidifiers, filtration systems, ductwork and air diffusers, heat source, controls and alarm systems.

3.8.2.2 Utilities (types): Electric service and emergency generators, high-pressure steam source, water supply (potable, chlorinated, acidified, demineralized, UV-sterilized, filtered).

3.8.2.3 Sterilizing equipment (types): High-vacuum, double door autoclave system, ethylene oxide, ultraviolet equipment, ionizing radiation source.

3.8.2.4 Mechanical washing equipment (types): Rack washer, tunnel washer, batch washer, bottle washer.

3.8.2.5 Water-dispensing equipment: Automatic distribution, chlorinators, filters, demineralizers, ultraviolet sterilizers.

3.8.2.6 Waste disposal: Incinerators, vacuum systems, mechanical disposal.

3.9 Classification of Barrier Systems Based on Method of Contamination Control.

3.9.1 The major operational variables in a barrier system are quality, quantity, and source of animals; frequency and method of introducing animals through the barrier; processing of materials through the barrier; entry of animal technicians into the barrier; method of housing and handling animals; the environmental systems, with special emphasis on the air-handling systems; and monitoring practices.

Type 1: Maximum-security barrier

1. Animal source -- defined microbially associated animals.

2. Animals are maintained in isolation and then introduced via a port system into the barrier.

3. Sterile materials, including cages, food, bedding, and other supplies enter the barrier without contamination.

4. All personnel entering the barrier must strip, shower, or pass through an air wash, wear sterilized uniforms, wear face mask, gloves, and hair and shoe covers.

5. All animals are transferred by forceps previously disinfected; manual handling is kept to a minimum.

6. Air supply is HEPA filtered (99.97 percent effective at 0.3 micron particle retention). Air recirculation is permitted if properly monitored.

Type II: High-security barrier

1. Animal source - barrier-maintained animals.

2. Animals are shipped in filter boxes and introduced via a secure port system (quarantine within the barrier is optional).

3. Materials - same as Type I.

4. Personnel - same as Type I.

5. Animal Care - same as Type I.

6. Air supply is filtered (95 percent effective at 0.3 μ). No air recirculation is permitted unless HEPA filtered.

Carcinogen Bioassay Program

Specification No. CBM-2

Subject: BARRIER FOR PREVENTION OF CONTAMINATION BY PATHOGENIC MICROORGANISMS	**Date:**	**Sheet** 5	**Of** 6

Type III: Moderate-security barrier

1. Animals are obtained from a reputable breeder and designated as barrier or monitored animals. Monitoring results are available for review in order to select suitable animals for research projects.

2. Animal entry - same as Type II, but each shipment should be placed in room containing animals from only one vendor.

3. Materials are either sterilized or sanitized or are heat-treated to kill all pathogenic vegetative microbial forms. If cages are sanitized instead of autoclaved, water temperature sensors that shut off the washing machine (less than 108°F) are recommended.

4. Personnel - same as Type I, but use of face masks and gloves may be modified.

5. Animal care - same as Type I or modified to include hand contact.

6. Air supply filtration is rated at 85 percent efficiency or better for 0.3 µ particle retention.

Type IV: Minimal-security barrier

1. Source of animals - same as for Type III, except that these are usually monitored animals held within a barrier. The supply colony may therefore have antibodies to known viral pathogens, and certain bacterial agents may be present. Knowledge of monitoring results is critical for selection and proper use of these animals.

2. Animals may be introduced via exit corridors, minimizing exposure. Containers do not enter rooms. Animals may be quarantined outside barrier then introduced via transport cages.

3. Materials - same as Type III.

4. Technicians enter through personnel lock, but security measures less stringent than Type III

5. Investigators abide by rules for animal techincians or have an option in some areas of the barrier to enter their own animal rooms from the exit corridor after donning disposable shoe covers and clean laboratory coats and then washing hands and using disposable gloves. They cannot enter other animal rooms or enter clean corridors.

Subject:	Date:	Sheet	Of
BARRIER FOR PREVENTION OF CONTAMINATION BY PATHOGENIC MICROORGANISMS		6	6

6. Animal handling - generally the same as Type III.

7. Air supply - same as Type III.

4. QUALITY CONTROL

4.1 Methods used in monitoring must include a thorough visual examination of the overall barrier system and its operating components, particularly of personnel involved in animal husbandry, cage sanitation, machine maintenance, and decontamination.

4.2. Monitoring procedures for Types I and II barrier systems should include statistically significant sampling by microbiologic, histopathologic, and physical methods.

4.3 Monitoring procedures for Type III barrier system are the same as Type I & II, but depth and breadth of monitoring practices are reduced.

4.4 Monitoring procedures for Type IV barrier system are the same as Type III, but may be further reduced. Level of monitoring must be adequate for the purpose of the experiment.

4.5 Perform serology on personnel for the presence of antibodies to animal viruses.

5. PACKAGING

Not applicable here.

6. NOTES

6.1 This specification is taken from: Long-Term Holding of Laboratory Rodents, ILAR News, Volume XIX, Number 4, 1976, L9-L12.

6.2 Calling a Type I barrier "maximum security" does not presuppose that contamination will not occur. Actual quality of the animals in such a system should be known and duly recorded.

Carcinogen Bioassay Program

Specification No. CBM-17

Type: MATERIALS SPECIFICATION	**Sheet** 1	**Of** 8
Subject: CHEMICALS FOR TESTING IN THE CARCINOGEN BIOASSAY PROGRAM		

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers chemicals to be tested for carcinogenic potential in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

2.1 Code of Federal Regulations, Title 42, Section 72.25, 1972.

2.2 Code of Federal Regulations, Title 49, Section 173, 1973.

3. REQUIREMENTS

3.1 All samples of chemicals to be tested for carcinogenic potential in the Carcinogen Bioassay Program shall be collected by the supplier in a manner that insures that the sample is representative of the entire batch or lot.

3.2 Chemicals to be tested will be specified and supplied to the Analytical Subcontractor and Bioassay Laboratory by Program Management (6.1, 6.2, 6.3).

3.3 Pure reference standards to be used in all relative purity assays as well as for comparison of different lots of chemicals shall be obtained from the National Cancer Institute, U.S. Pharmacopeia, National Formulary, commercial sources, or shall be prepared by the Analytical Subcontractor (6.3).

3.4 The homogeneity, chemical identity, impurity content, stability, and storage parameters of each test chemical shall be determined prior to its bioassay by the Analytical Subcontractor. Results shall be given to the bioassay laboratory as well as to Program Management (6.1, 6.2, 6.3).

3.5 Identification and quantification of impurities as well as purification of the test chemical may be necessary in some instances (6.1, 6.3).

3.6 Homogenization of test chemical (6.3)

3.6.1 Samples of the test chemical shall be ground in a Fitz

Mill, homogenized in a Day Blender, and then analyzed for homogeneity by the Analytical Subcontractor. Samples shall be taken at three levels in the blender for analysis.

3.6.2 The entire batch of chemicals to be used by the bioassay laboratory shall be ground and homogenized by the Analytical Subcontractor.

3.7 Identification (6.3)

3.7.1 Single compounds

Two or more of the following tests shall be used depending on the amount of sample available, nature of the compound, and the number of techniques necessary to identify the compound:

3.7.1.1 Spectral data

- Infrared
- Ultraviolet
- Visible
- Nuclear Magnetic Resonance
- Mass Spectroscopy - when necessary to clarify structural data.

3.7.1.2 Physical constants

- Melting Point
- Boiling Point
- Refractive Index
- Optical Rotation
- Elemental Analysis

3.7.1.3 Chromatography

- Thin-Layer - all but highly volatile cmpds
- Vapor-Phase - highly volatile compounds
- High-Pressure Liquid - non-volatile polar compounds
- Gel Permeation Mtds. - non-volatile polar compounds

3.8 Assay (6.3)

3.8.1 Single Compounds

3.8.1.1 Assay methods for the test chemical shall be determined by the Analytical Subcontractor on the basis of chemical nature of the compound and the procedure by which it was synthesized.

3.8.1.2 Two or more of the following methods shall be used for each chemical depending on the amount of sample available, nature of the compound, and number of procedures necessary to determine the level of purity.

3.8.1.2.1 Elemental Analysis

3.8.1.2.2 Chromatography - as for 3.7.1.3

3.8.1.2.3 Spectroscopy

- Emission
- Visible
- Ultraviolet
- Infrared
- Fluorescence
- Nuclear Magnetic Resonance
- Electron Spin Resonance
- Mass Spectroscopy

3.8.1.2.4 Titrimetry and Electroanalysis

- Colorimetry
- Potentiometry - Compounds with reactive groups, e.g., amines, acids, oxidizable, reducible groups, etc.
- Polarography - Reducible compounds
- Voltammetry - Oxidizable compounds
- Coulometry
- Amperometry

3.8.1.2.5 Absolute Purity Analysis - Reference standards and compounds where high purity is critical

- Differential Scanning Colorimetry
- Phase Solubility

3.8.2 Mixtures (6.3)

3.8.2.1 Isolation of Components

At least two of the following methods shall be used:

- Crystallization
- Preparative Chromatography
  - Thin-Layer Chromatography
  - Column Chromatography - Preliminary to high pressure liquid or vapor-phase for solid compounds
  - Vapor-Phase Chromatography - Volatile compounds
  - High-Pressure Liquid Chromatography
- Spinning Band Distillation - Volatile compounds
- Zone Refining - Solid compounds
- Sublimation - Solid compounds

3.8.2.2 Identification of Components - As for 3.7

3.8.2.3 Quantification of Components - As for 3.8

3.9 Reanalysis (6.2)

A sample of the bulk test chemical shall be analyzed for purity at various intervals by the bioassay laboratory, or by a subcontractor in close proximity to the laboratory so that the analytical results are available within one week. Analytical methods to be used will be provided by the Analytical Subcontractor.

3.9.1 Each chemical lot shall be reanalyzed for purity at four-month intervals from receipt of the lot through the subchronic test.

3.9.2 Each batch of chemical to be used for the chronic test shall be analyzed for purity two weeks prior to initiation of the test, during the test at three, six, twelve, and eighteen months, and within two weeks after sacrifice of the last treatment group.

3.9.3 If a new lot of chemical must be used after beginning of the chronic test, it shall be analyzed immediately, and thereafter at the same times the initial batch would have been analyzed.

3.9.4 Any significant change in purity or appearance of the test chemical shall be reported to Program Management immediately via telephone by the Principal Investigator (6.2).

3.10 Stability and Storage

3.10.1 Stability (bulk and solution) and storage parameters for each test chemical - with respect to temperature, light, air, and moisture -

shall be determined prior to its bioassay by the Analytical Subcontractor (6.1, 6.2, 6.3).

3.10.2 Light-sensitive chemicals shall be stored in the dark in amber bottles. All work with such chemicals shall be performed in a darkened room with filters to exclude ultraviolet light.

3.10.3 Bulk stability shall be determined at 0°C, 25°C, and 60°C for periods up to two months; and decomposition shall be followed by analytical techniques (6.1, 6.2, 6.3).

3.10.4 Each test chemical shall be handled and stored by the bioassay laboratory in accordance with directions provided by the Analytical Subcontractor (6.2, 6.3).

3.11 Purification (6.3)

3.11.1 Chemicals requiring purification prior to the bioassay shall be subjected to treatment appropriate for the chemical nature of the mixture and required purity of the test chemical.

3.11.2 The following techniques are to be used singly or in combination:

- Crystallization
- Preparative Chromatography
  - Thin-Layer - For all but highly volatile compounds
  - Column - Preliminary method for solid compounds
  - Vapor Phase - Volatile products
  - High-Pressure Liquid - Non-volatile polar compounds
  - Spinning Band Distillation - Volatile products
  - Zone Refining - Solids
  - Sublimation - Solids

3.11.3 Following purification, the test chemical shall be analyzed as indicated in 3.8.

3.12 Disposal of Residual Chemicals (6.2)

3.12.1 All test chemicals shall be retained by the bioassay laboratory until directed by Program Management to ship the materials to the Analytical Subcontractor.

3.12.2 All chemicals shall be packaged and shipped in accordance with (5).

4. Quality Control

4.1 Bioassay tests shall be performed only with chemicals which meet project identity, purity, and stability standards as indicated by the Analytical Subcontractor's testing results.

4.2 The supplier shall certify that all samples submitted to Program Management were collected in accordance with project specifications.

4.3 Sample Storage, Labeling and Records

4.3.1 All samples shall be logged in upon receipt with the following information: log number, identification of material, purchase order number, manufacturer, date.

4.3.2 Log number and shelf-life expiration date shall be added to the manufacturer's label on all containers.

4.3.3 The Quality Control Supervisor shall make certain that all test chemical samples are stored in accordance with recommendations of the manufacturer and Analytical Subcontractor.

4.4 Identification and Quantitation

4.4.1 Samples which do not meet all project identification criteria shall be considered unacceptable for bioassay testing.

4.4.2 Quantitative assays in which reference standard results differ by more than 10% from the certified value shall be considered invalid and must be repeated.

4.4.3 Samples shall be rejected if:

4.4.3.1 Percentage of main ingredient differs from project specifications by 10 or more percent.

4.4.3.2 Impurities, other than those indicated acceptable in project specifications, are found.

4.4.3.3 Any contaminant exceeds the maximum acceptable concentration according to project specifications.

4.4.3.4 The Analytical Subcontractor deems that the sample can be satisfactorily purified to meet project specifications. The sample may be accepted provisionally under these conditions.

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4.4.4 All assays of purified samples, stability tests and reanalyses shall be subjected to the same controls indicated in 4.4.1 - 4.4.3 above.

4.4.5 All identification and quantitation review results and actions shall be recorded in the Quality Control Record Book and signed by responsible personnel.

4.5 Storage Control

4.5.1 Storage areas for test chemicals shall be equipped with automatic temperature and humidity regulators connected to an automatic alarm system.

4.5.2 The Quality Control Supervisor shall make certain that all environmental storage parameters (3.10.1) are checked periodically and that any indicated adjustments are made promptly.

4.5.3 All outdated test chemicals shall be withdrawn and disposed of as indicated by Program Management (3.12).

4.6 Equipment Control

4.6.1 All equipment shall be inspected at intervals recommended by the manufacturer. Cleaning and all other stipulated maintenance operations shall be performed as scheduled. Defects shall be repaired properly.

4.6.2 Precision instruments shall be recalibrated at intervals and by procedures, recommended by the manufacturer.

4.6.3 All inspections, maintenance operations, and recalibrations shall be recorded in the Quality Control Record Book and signed by the responsible personnel.

4.7 Reagents Control

4.7.1 Packing slips accompanying all reagent shipments shall be examined for conformance with project specifications. Reagents which differ significantly from project specifications shall be rejected.

4.7.2 All reagents shall be performance tested upon receipt and at stated intervals during storage. Reagents giving substandard performance shall be returned to the supplier or discarded.

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4.7.3 All results and actions involved in reagents control shall be recorded in the Quality Control Record Book and signed by responsible personnel.

5. Packaging

5.1 Stable carcinogens shall be packaged and shipped in accordance with regulations of the Department of Health, Education, and Welfare for the transportation of etiological agents (2.1).

5.2 Unstable chemical carcinogens (corrosive, explosive, flammable) shall be packaged and shipped according to Department of Transportation regulations (2.2).

6. Reference Documents

6.1 Guidelines for Carcinogen Bioassay in Small Rodents, NCI-CG-TR-1, Sontag, J.M., N.P. Page, and U. Saffioti, National Cancer Institute, DHEW, Bethesda, Maryland, February 1976.

Carcinogen Bioassay Program Specification No. CBT-1

Type: STANDARD METHODS OF TEST				**Sheet** 1	**Of** 2
Subject: PURITY TESTS ON CHEMICALS FOR STUDY IN THE NCI CARCINOGEN BIOASSAY PROGRAM					
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers tests for purity on chemicals selected for study in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Each chemical to be studied in the Carcinogen Bioassay Program shall be tested for purity prior to its bioassay by the program management analytical subcontractor.

3.2 Purity tests shall be designed to:

3.2.1 Confirm identity of the test chemical.

3.2.2 Determine concentration of test agent in bioassay batch.

3.2.3 Characterize each contaminant encountered physically (e.g., chromatographic behavior).

3.2.4 Identify major or critical contaminants and, in some cases, determine percentage of each, if requested by program management (2.1).

3.3 Purification of test chemical may be necessary in some cases.

3.4 Chemicals will not be released for bioassay until analytical results indicate that the chemical is of sufficient purity.

3.5 The bioassay test laboratory shall reanalyze the chronic test chemical batch for purity two weeks prior to the start of test and at three, six, twelve, and eighteen months during the bioassay, as well as within two weeks after sacrifice of the last treated group. The analytical methods will be supplied by the analytical subcontractor.

3.6 If a new batch of chemical must be used after initiation of the

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chronic test, its purity shall be tested immediately upon receipt and thereafter at the same times indicated for the initial batch.

3.7 Purity analysis results shall be reported to the Principal Investigator immediately and no later than four weeks to program management. Reports shall include methodology and critical raw data (spectra, chromatographic traces), analysis and interpretation of the data, and conclusions. The report shall be signed by the responsible chemist.

3.8 Any significant changes in purity of the test chemical during the bioassay shall be reported immediately to program management via telephone by the Principal Investigator.

4. QUALITY CONTROL

4.1 All analytical instruments used in purity tests on chemicals to be studied in the Carcinogen Bioassay Program shall be recalibrated monthly. All recalibration data shall be recorded in a bound notebook, dated, and signed by personnel involved.

4.2 Standard reference samples of known purity supplied by the manufacturer shall be run in parallel with test chemicals in all purity tests.

5. PACKAGING

Not Applicable

6. NOTES

6.1 Two methods generally will be used in reanalysis for purity of the test chemical. The methods shall be pertinent to the chemical and its suspected degradation products. The methods also should be complementary and as simple as possible. The purity of a volatile liquid, for example, might be checked by gas-liquid chromatography and a spectroscopic technique.

7. REFERENCE DOCUMENTS

7.1 Guidelines for Carcinogen Bioassay in Small Rodents, NCI-CG-TR, Sontag, JM., N. P. Page, and U. Saffiotti, National Cancer Institute, National Institutes of Health, Bethesda, Md., 1976.

2.6.2 Acceptance Specifications

The acceptance specification, the next important part of the laboratory's quality control system, should spell out the sampling to be done on receipt of the chemical, the analyses to be performed, and should state the acceptance criteria. If further purification is required before use in a bioassay, the purification steps should be specified.

A sample should be representative of the lot as received. The general principles of sampling are covered in Section 2.3 in the context of the larger task of the laboratory to perform sampling and measurement at all stages of research.

The laboratory operating protocol should contain specifics regarding the analyses to be performed at the materials acceptance stage. These will include: identity of the material (qualitative analysis), purity, identity of impurities, percent of each impurity (quantitative analysis), and general nature of unidentified impurities. Also, it is important that possible contaminants, if they could have an adverse effect on the experiment, be shown to be absent. In identifying the impurities all should be characterized physically, as by chromatographic behavior, and the major ones should be directly identified.

By acceptance criteria are meant the rules for accepting or rejecting a lot for failure to meet specification. In general these criteria are expressed as plus and minus ranges about the nominal quality beyond which results are to be judged inacceptable. These plus and minus limits are statistically calculated confidence limits obtained from repetitive measurements of the same sample.

Filter media can be classified as reagents (U.S. EPA, 1973b) The purchase specification should include requirements for flow characteristics, surface uniformity, occurrence of pinholes, pH, ion blanks, and light reflectance or transmittance.

Incoming lots should be sampled and tested for measurable characteristics. Attributes sampling (for example for pin hole leaks in glass fiber filters) may not be describable because each filter should be examined before use in the field.

2.6.3 Control of Chemicals and Reagents

The purchase specification or purchase order should instruct vendors to mark individual containers and packing slips with name of material, vendor's name and address, vendor's lot number, quantity, and material specification number and date.

Upon receipt, the package marking or packing slip should be checked against the purchase order. Discrepancies will subject the lot to rejection. If it is desired to check the validity of the certification, or if intended use requires acceptance sampling and testing, it is done at this time. The material is then logged in. The log sheet should have the following columns: Assigned log number, identification of the material, purchase order number, name of vendor, date, and disposition (accepted or rejected). The label of each container should be marked with the log number and the shelf-life expiration date. Shelf-life, particularly of biological reagents, is usually determined by the vendor and included on the container label. The inventory of chemicals and reagents should be checked monthly to identify materials approaching the shelf-life expiration date.

The disposition record may be used to establish trends in vendor performance and may indicate a need to clarify specifications or change vendors. If purity tests are made, the record of these tests may be charted providing another opportunity to keep an eye on quality variations. A check on quality, strength, concentration and composition of chemicals and reagents is usually made as part of the analytical procedure as a precaution against omissions in the acceptance procedure.

Storage of chemicals and reagents should be under conditions to minimize deterioration with time. A first in, first out inventory policy should be applied.

Reagents must be prepared and standardized with utmost care. Written procedures should be available in the laboratory.

Standard solutions will require occasional restandardization. Storage and standardization requirements for several standard solutions are given in Table 2.8 (U.S. EPA, 1973b).

Labels on standard solution bottles should include chemicals used, manufacturers, lot numbers, date of preparation, date of next standardization, standardization conditions of analysis (temperature, pressure and humidity).

Standard reference materials are available for many chemicals from the National Bureau of Standards. The availability of primary standards, particularly of biological materials may be limited and commercial manufacturers must be depended upon. Standard reference materials are used for standardizing solutions, calibrating equipment and monitoring precision and accuracy of measurement methods. Supplier's recommended storage and handling procedures should be followed.

TABLE 2.8 RESTANDARDIZATION REQUIREMENTS
(U.S. EPA, 1973b)

Solution		Storage Requirements	Frequency of Restandardization
0.02-1N	Sodium hydroxide	Polyolefin	Monthly
0.02-1N	Hydrochloric acid	Glass	Monthly
0.02-1N	Sulfuric acid	Glass	Monthly
0.1N	Iodine	Amber glass Refrigerate	Weekly (open bottles) Monthly (sealed bottles)
0.1N	Sodium thiosulfate	Glass	Weekly
0.1N	Ammonium thiocyanate	Glass	Monthly
0.1N	Potassium bichromate	Glass	Monthly
0.1N	Silver nitrate	Amber glass	Monthly
0.1N	Potassium permanganate	Amber glass	Weekly

2.7 CONTROL OF TEST SUBJECTS

2.7.1 Control of Animal Breeding

Quality control must begin in the breeding and production colonies. Since in most instances, biological research laboratories purchase animals from outside suppliers, it is important that quality control requirements be spelled out in purchase contracts. The detail required in contracts increases if the supplier does not have a good reputation for quality or if he cannot produce evidence that he maintains an adequate quality control program. It is even more important that quality requirements be very specific if the laboratory is contracting for purchase of animals (such as primates) caught in the wild.

All the requirements for Good Animal Care Laboratory Practices (see following Section and Appendix B) apply and in addition to requiring conformance to the BLP's, the contract may specify the following taken from a contract for supply of Sherman stock rats used by the Health Effects Research Laboratory, Research Triangle Park:

- The Contractor shall maintain a production colony under barrier conditions in accordance with standard industry practices (Reference: Defining the Laboratory Animal, National Academy of Sciences, Washington, D.C., 1971).

- The Contractor shall re-certify the continued absence of known pathogens in the production colony every six (6) months for the duration of this contract. Such certification shall include as a minimum, lists of tests used and results for the following pathogens: viral, PVM, Reo 3, GDVII, KRV, H-1, Mse, Adeno, MHV, LCM, RCV, Sendai, bacterial, mycoplasma pulmonis, bordetella bronchiseptica, pseudomonal aeruginosa, salmonella typhimurium, coryne bacterium kutsheri, streptobacillus moniliformis, bacillus piliformis, and pasteurella pneumontropica. In addition, animals shall be free of arthropod and helminth parasites known to infect this species (rats).

- The Contractor shall re-derive replacement breeding stock as often as necessary to maintain the quality of animals specified in this contract.

- The Contractor shall group-house the holding stock animals with three to five animals per cage. All such animals shall be held in stock until shipment is requested by the Project Officer or his designated representative. Animals over 90 days of age shall be disposed of by, and at the discretion of the Contractor. All animals shall be housed in existing Contractor-owned and-operated facilities. All testing of animals, to ascertain their quality, shall be done by Contractor personnel in the Contractor's own laboratories.

The National Academy of Sciences published a series of Procurement Specifications (Contract Clauses) for experimental animals including:

- Conditioned Random-Source Dogs, 1968
- Conditioned Random-Source Cats, 1968
- Kennel-Produced Dogs, 1969
- Colony-Produced Cats, 1969
- Defined Laboratory Rodents and Rabbits, 1973
- Defined Wild Caught Old World Monkeys.

2.7.2 Good Animal Care Laboratory Practices

The basic references for good animal care are U.S. DHEW (1974) and Sontag et al. (1976). In addition, the FDA Regulations (FDA, 1976) have had a substantial impact on thinking about improvement in non-clinical laboratories.

A complete set of Good Animal Care Laboratory Practices suitable for mammalian bioassay with rodents is given in Appendix B.

2.8 CONTROL OF PERFORMANCE OF EXPERIMENTS

2.8.1 Quality Control Charts

The control chart is a graphic means of analyzing data and of controlling the consistency of results over time. The basic concept on which the control chart is based is that the random variations to which all measurements are subject occur over short periods of time; on the other hand, special causes of variation, for which an assignable cause may be found, occur over relatively longer periods of time. Therefore, control limits are calculated from the average **variation within small sets or** subgroups of data collected essentially at the same time. The limits are used to control the variation of subgroup averages over time. This is possible because the variance of an average is related to the variance of the individual measurements inversely as the number of measurements averaged:

$$s^2 \text{ (average)} = s^2 \text{ (Individuals)}/\ n,$$

and therefore, $s_{\bar{x}} = s\ /\ \sqrt{n}$.

If the control limits are exceeded, a signal is given that a non-random event has occurred.

This gives to control limits an entirely different significance than that of confidence limits as calculated using Eq. 2.2.3. Confidence limits are calculated from the whole set of data and include both short-term random variation and any longer-term nonrandom variation that may have occurred while the data were being collected. Confidence limits are calculated as though all variation was random but, since an internal check of randomness may not have been made, this may not be the case. Usually, the variance calculated from the whole of a set of data is larger than the variance calculated by control chart techniques using the same data but arranging them in subgroups. Charts on which confidence limits were plotted would be useless for control.

If there are no special assignable causes of variation in a set of data the variance in the long-term should not be significantly different from the average variance within short-term subsets of the data. Then the measurement system is said to be in a state of control. Only random causes affect the variance and there are no perturbations. In the controlled state, confidence limits calculated from the whole set of data should be very close to the control limits.

Control charts are used to prevent persistence of assignable causes of variation, such as operator error, instrument drift, changes in reagents, or environmental effects, by providing a visual signal when something non-random has occurred. If a point goes out of control (is outside the control limits) when plotted on the control chart, action should be taken to identify and correct the cause. The limits are placed (usually at plus and minus three standard deviations from the average of the measurements) so that it is very unlikely that a departure from the limits could have been caused by chance alone. Therefore, it is worth while to look for the cause of trouble every time the measurement process goes out of control. As originally proposed by

Shervhart (1931), the control chart was intended for economic control, i.e., effort would be spent on trying to identify assignable causes of variation only infrequently when actually variation was only random.

The control chart method can be used to analyze any set of data, even small sets usually associated with biological experimentation. It takes larger sets of data (small sets gathered over a period of time) to make the control chart work well for control or for improvement of an experimental procedure.

One of the advantages of the control chart, which makes it attractive for analysis as well as control of data, is that the variance on which the limits are based is calculated using the range (difference between the largest and smallest number in a small set) rather than the mean square variation. This lessens the calculation load because the arithmetic is simpler. In addition, the control chart calculation provides a within-group/between group comparison of variation which is easier than the formal analysis for variance. Thus, single factor experiments (the kind most frequently met with in biological research) could be analyzed using the control chart technique rather than by the methods illustrated in Section 2.2.

The selection of the small sets, or subgroups, of the data must be made on a rational basis. For example, it is rational to try to control measurement systems by making replicate tests (two or more) on standard samples on a periodic basis. The control limits are based on the average variance within the replicate subgroups and the averages of successive replications are plotted. The rationale is that it is worth trying to control the test over a period of time (differences between the averages) as closely as possible to control the differences within the replicate subgroups.

A generalized control chart for averages of small subgroups of data is given in Figure 2.2.

The central line on the chart is the grand average of all the available data. A minimum of 10 subgroups of data should be available before plotting of a control chart is attempted. It is necessary to have about 30 subgroups before the limits can be adopted as standard control limits.

Three standard deviation limits (3-sigma limits) are generally used. The formula for the control limits for a control chart for subgroup averages is:

$$\bar{\bar{X}} \pm A_2\bar{R}$$

where $\bar{\bar{X}}$ is the grand average of all the data, $\bar{R}$ is the average range of the subgroups, and A_2 is a factor for 3-sigma limits for subgroups of a given size, available in any quality control text book.

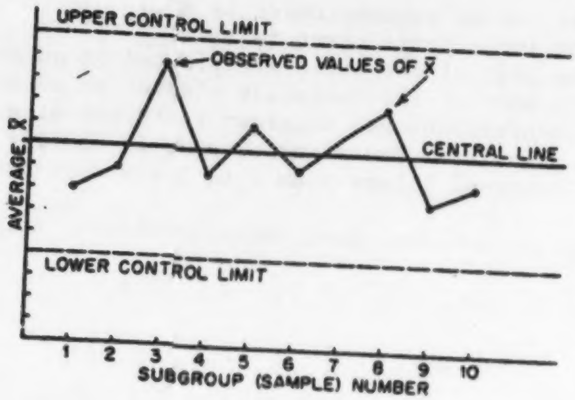

Figure 2.2 Generalized control chart for averages.

It is also possible to plot a control chart for ranges to control the variability within the subgroups (i.e., the difference among replicates). The formula for limits for ranges are:

Lower limit: $D_3\bar{R}$

Upper limit: $D_4\bar{R}$

where $\bar{R}$ is the average range of the subgroups and D_3 and D_4 are factors for 3-sigma limits. These limits are plotted below and above a central line plotted at $\bar{R}$. They are non-symmetric about $\bar{R}$.

The horizontal scale on the chart is the subgroup number. The vertical scale is a measurement scale.

Averages of subgroups of the data are plotted, usually in time sequence, so that the occurrence of a point out of control may be identified by the time it occurred.

A convenient format for recording of data and calculations follows:

Subgroup No	Observations					Average $\bar{X}$	Range R
	1	2	3	4	5		
1							
2							
3							
.							
.							
.							
etc.							

Totals

SS_1 $\bar{\bar{X}} = \Sigma\bar{X}$ / No. subgroups

SS_2 $\bar{R} = \Sigma R$ / No. subgroups

The central line for an average chart is SS_1.
The central line for a range chart is SS_2.
The $\bar{X}$'s from the body of the table are plotted on an average chart.
The R's from the body of the table are plotted on a range chart.

The number of observations per subgroup (subgroup size = n) determines the values of A_2, D_3, and D_4. The subgroup size is usually small. Experience has shown that subgroup sizes from 2 to 5 are most used.

A partial table for control chart factors follows (ASTM, 1976):

Subgroup Size (n)	A_2	D_3	D_4	d_2
2	1.880	0	3.267	1.128
3	1.023	0	2.575	1.693
4	0.729	0	2.282	2.059
5	0.577	0	2.115	2.326
6	0.483	0	2.004	2.534
7	0.419	0.076	1.924	2.704
8	0.373	0.136	1.864	2.847
9	0.337	0.184	1.816	2.970
10	0.308	0.223	1.777	3.078

Values for the factor, d_2, are given above because this factor is useful in estimating the standard deviation from the range as follows:

$$s = \bar{R}/d_2$$

Using this relationship, the precision of a method can be calculated from the average range of successive replicate determinations on a standard material, as:

$$P = \pm\, t\, \bar{R}/d_2$$

2.8.2 Assessing Laboratory Pe formance

2.8.2.1 Precision --

For control of precision of results, replicate measurements on a standard material are made periodically (e.g., daily) by the operator. When ten sets of replicates are available, tentative control limits for averages (and for ranges, if desired) are calculated and an average chart is constructed as explained above. The ten averages are plotted on this chart. The limits are extended over more daily periods and an additional point is put on the chart daily, as the tests are completed. A point out of control means that something unusual has occurred and that it is worthwhile to look for a cause. The cause may be an operator error, a change in reagents, instrument malfunction, a change in the environment, or some other identifiable change in the procedures. If precision is to be maintained, corrective actions must be taken.

When the average chart is in control, the control limits may be used to express the precision of the average of replicates:

$$P_{\bar{x}} = \pm A_2\bar{R}$$

The precision of the method is usually expressed in terms of individual measurements, so if the average control chart is in control,

$$P = \pm A_2\bar{R}\sqrt{n}$$

where n is 2, for duplicate daily determinations.

Relatively infrequently, something can happen to the replication of measurement causing the range to be larger than usual. If the operator is new to the method, it may be desirable to plot the ranges of the replicates, at least until it is evident that his skill is sufficient to warrant dropping the range chart.

2.8.2.2 Accuracy--

Accuracy may be controlled using spiked samples. Percent recovery is determined on two or more samples at periodic intervals, say daily. Control charts for averages (and perhaps ranges) are set up, as for control of precision.

Action should be taken daily (or whenever percent recovery is determined) to keep this chart in control. When the accuracy control chart is in control, the control limits may be used to develop a statement about accuracy of the method. As defined in Section 1, accuracy is made up of the bias (or constant error) of the average of a number of measurements from the known amount added to the spiked samples plus uncertainty of the average. Therefore, accuracy is better pinned down when the number of measurements in the average is larger. We would express it using all the information available to us. The limits on the average chart apply to averages of the periodic replicate determinations of percent recovery.

Using the control limits of a measurement process that is in control, accuracy of the method is expressed by:

$$A = \left|\bar{\bar{X}} \pm A_2\,\bar{R}\,\sqrt{n/N}\right|$$

where n is 2 for duplicate periodic determinations and N is the total number of measurements at hand and used to calculate $\bar{R}$.

It is possible, in both the precision and accuracy control chart procedure, to use subgroups of varying size. This complicates the calculations but it can be handled using methods available in quality control texts (Juran, 1974; Duncan, 1965; Grant and Leavenworth, 1972).

For further application of control charts in the environment and related areas see U.S. EPA, 1972, 1973b; NIOSH (undated).

2.9 INTERLABORATORY TESTING

After a laboratory has brought its measurements under control, it is desirable to extend the efforts at improving laboratory performance to checking with other laboratories to see what can be done to improve agreement of results between laboratories.

This is usually done using standard samples. These standard samples are often prepared by a reference laboratory and distributed according to plan among participating laboratories. Large organizations, such as EPA, may use one of its laboratories as the reference laboratory and send samples to all in-house and contractor laboratories.

Replicate results from each of a number of laboratories may be analyzed using analysis of variance (single factor model). Also, range control charts can be used to compare the variance within the laboratories. If the within-laboratory ranges are in control, an average control chart can be used to plot laboratory averages using the grand average of all laboratories as the center line. Limits would be based on the average within laboratory ranges and the points plotted would be the laboratory averages.

Correction of points out of control on the range charts would be the responsibility of the individual laboratory because they represent internal laboratory problems.

If the average chart is out of control the reason may be different instrumentation in the different labs, different degrees of environmental control (i.e., temperature, humidity, etc.), or differences in methods or in the closeness with which standard methods are followed. Moreover, some labs may be out of control on the high side and some on the low. Bringing the laboratory community into line requires collaborative effort. Some causes of failure to compare well with the average may be correctable and some not. However, experience has proved that it is well worthwhile to do this kind of proficiency testing because many problems require comparison of results from more than one laboratory. These comparisons cannot be made with confidence unless the laboratories involved have internal control and there is some consistency in testing the same thing in different laboratories.

When an interlaboratory testing program results in evidence of control between laboratories, some kind of a calculation can be made of the precision and accuracy of measurement methods in the making of interlaboratory comparisons. Until such is the case there must remain some doubt about apparent differences between laboratories.

2.10 DATA HANDLING AND REPORTS

The quality of the output of research, the data and the reports, is what the whole quality assurance activity is about. By controlling all the elements of work, assurance is given that the results are valid and scientifically defensible. At the data-handling stage, research management must take steps to preserve the integrity of the results achieved.

This begins with the planning for data collection using formats or forms which are clear, complete, and designed to limit human errors of entry, transcription and use. Some examples of data forms in use are illustrated in the Sections dealing with the various areas of research. Much data is still entered manually so that these forms should be helpful. Increasingly, data are collected and organized in automated systems. These systems are usually designed for a particular purpose in a unique laboratory situation. The importance of good programming for such a system cannot be overemphasized.

The forms for manual data entry may be loose-leaf. However, for both field and laboratory research programs it is highly desirable that the data be recorded, at least originally, in bound notebooks. It is good practice to require strict adherence to the established custom of having the entries in the notebook signed by the person taking the data and witnessed periodically, preferably daily, by the supervisor. If the experimenter is the senior individual in the laboratory it is good practice to have the entries witnessed by an associate. Although this may appear to be a stricture on the research task, it is extremely important if the work is later likely to be subjected to any kind of litigation.

It is desirable to have all records under control (a sort of "Chain of custody" of records) which means that notebooks should be numbered, issued centrally, and returned to a designated repository when filled or at the end of a project. There should be written instructions on the retention period for records and how they shall be filed and stored.

In some very large research projects, responsibility for design, conduct, analysis and reporting of the work may be fragmented among sponsors, contractors, and subcontractors. The problems of maintaining validity and integrity of data may be amplified under such conditions but it is not the intent of the Guidelines to address the managerial problems encountered. Much research is still done by smaller laboratories, or groups of laboratories, or by individual researchers. The responsibility for report preparation is localized. Formal reports should be required. These reports should be subjected to review within the laboratory. If the work is to be published, the major journals require further review by peers in the same area of research. One of the major requirements of good scientific work is that it should be verifiable. This requires that all the pertinent data must be reported and that methodology should be well enough described so that the experiment could be reconstructed independently.

2.11 REFERENCES

ASTM. 1974. Book of ASTM Standards, Part 23, Water; Atmospheric Analysis. American Society for Testing and Materials, Philadelphia, Pa.

ASTM. 1976. Manual on Quality Control of Materials. STP 15-D. American Society for Testing and Materials, Philadelphia, Pa.

ASTM. 1977. Precision and Accuracy as Applied to Measurement of a Property of a Material. E 177-71. Recommended Practice for Use of the Terms. Annual Book of ASTM Standards, Part 41. American Society for Testing and Materials, Philadelphia, Pa.

Armitage, P. 1971. Statistical Methods in Medical Research. John Wiley and Sons, New York, N.Y.

Bennett, C. A., and N. L. Franklin. 1954. Statistical Analysis in Chemistry and the Chemical Industry. John Wiley and Sons, New York, N.Y.

Bicking, C. A. 1968. Sampling. In: The Encyclopedia of Chemical Technology. 2nd Edition, Volume 17. Interscience Publishers, pp. 744-762.

Bicking, C. A. 1976. The Sampling of Biological Materials. In: Proceedings of a Seminar on Sampling of Bulk Materials, European Organization for Quality Control. STAQUAREL 1976, Prague, Czechoslovakia.

Bicking, C. A., and F. M. Gryna, Jr. 1974. Process Control by Statistical Methods. In: Quality Control Handbook. 3rd Edition. J. M. Juran, F. M. Gryna, Jr., and R. S. Bingham, Jr. (eds.). McGraw-Hill Book Co., New York, N.Y.

Bliss, C. I., and R. A. Fisher. 1953. Fitting the negative binomial distribution to biological data. Biometrics 9: 176-200.

Cochran, W. A., and G. M. Cox. 1950. Experimental Designs. John Wiley and Sons, New York, N.Y.

Cox, D. R. 1970. Analysis of Binary Data. Methuen and Co., Ltd., London, England.

Cox, D. R. 1972. Regression models for life tables. J. Roy. Statist. Soc., Ser. B 34: 187-220.

Duncan, J. 1965. Quality Control and Industrial Statistics. 3rd Edition. Richard D. Irwin, Inc., Homewood, Ill.

Eisenhart, C. 1947. The assumptions underlying the analysis of variance. Biometrics 3: 1-21.

Finney, D. J. 1964. Statistical Methods in Biological Assay. 2nd Edition. Hafner Publishing Co., New York, N.Y.

Fisher, R. A. 1925. Theory of statistical estimation. Proc. Cambridge Phil. Soc. 22: 700-725.

Fisher, R. A. 1947. The Design of Experiments. 4th Edition. Oliver and Boyd, Edinburgh, England.

Fisher, R. A., and F. Yates. 1949. Statistical Tables for Biological, Agricultural and Medical Research. 3rd Edition. Hafner Publishing Co., New York, N.Y.

FDA. 1976. Proposed regulations for good laboratory practices. Food and Drug Administration, Fed. Regist. 41 (225) : 51206-51230. November 19, 1976.

Geldreich, E. E. 1975. Handbook for Evaluating Water Bacteriological Laboratories. 2nd Edition. U.S. Environmental Protection Agency, EPA-670/9-75-006.

Grant, E. L., and R. S. Leavenworth. 1972. Statistical Quality Control. 4th Edition. McGraw-Hill Book Co., Inc., New York, N.Y.

Juran, J. M. 1974. Quality Control Handbook. 3rd Edition. J. M. Juran, F. M. Gryna, Jr., and R. S. Bingham, Jr. (eds.). McGraw-Hill Book Co., Inc., New York, N.Y.

Kaplan, E. L., and P. Meier. 1958. Nonparametric estimation from incomplete observations. J. Amer. Statist. Ass. 53:457-481.

Kempthorne, O. 1952. The Design and Analysis of Experiments. John Wiley and Sons, New York, N.Y.

Miller, R. G., Jr. 1966. Simultaneous Statistical Inference. McGraw-Hill Book Co., Inc., New York, N.Y.

National Bureau of Standards. 1977. The International System of Units (SI). NBS Special Publication 330. U.S. Department of Commerce.

NIOSH. Industrial Hygiene Service Laboratory Quality Control Manual, Technical Report No. 78. National Institute for Occupational Safety and Health (NIOSH), Division of Laboratories and Criteria Development, Cincinnati, Ohio.

Rand, M. C., A. E. Greenberg, and M. J. Taras (eds.). 1975. Standard Methods for the Examination of Water and Wastewater. 14th Edition. American Public Health Association, American Water Works Association, and Water Pollution Control Federation. Washington, D.C.

Shervhart, W. A. 1931. Economic Control of the Quality of Manufactured Products. Van Nostrant, New York, N.Y.

Slack, K. V., R. C. Averett, P. E. Grewson, and R. G. Lipscomb. 1973. Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples. Book 5. U.S. Department of the Interior, Washington, D.C.

Sokal, R. R., and F. J. Rohlf. 1969. Biometry, The Principles and Practice of Statistics in Biological Research. W. H. Freeman and Co., San Francisco, Calif.

Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U.S. Department of Health, Education and Welfare, NIH 76-801.

Tarone, R. E. 1975. Tests for trend in life-table analysis. Biometrika 62:679-682.

U.S. DHEW. 1974. Guide for the Care and Use of Laboratory Animals. U.S. Department of Health, Education and Welfare, NIH 74-23.

U.S. EPA. 1972. Handbook for Analytical Quality Control in Water and Wastewater Laboratories. U.S. Environmental Protection Agency, Analytical Quality Control Laboratory, Cincinnati, Ohio.

U.S. EPA. 1973a. Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents. C. I. Weber (ed.). U.S. Environmental Protection Agency, EPA-670/4-73-001, Cincinnati, Ohio.

U.S. EPA. 1973b. Quality Control Practices in Processing Air Pollution Samples. U.S. Environmental Protection Agency, APTD-1132, Research Triangle Park, N.C.

U.S. EPA. 1974a. Pesticide Residue Analysis in Water: Training Manual. U.S. Environmental Protection Agency, EPA-430/10-74-012.

U.S. EPA. 1974b. Methods for Chemical Analysis of Water and Wastes. U.S. Environmental Protection Agency, EPA-625/6-74-003, Cincinnati, Ohio.

U.S. EPA. 1975. Model State Water Monitoring Program. U.S. Environmental Protection Agency, EPA-440/9-74-002.

U.S. EPA. 1976. Handbook for Sampling and Sample Preservation of Water and Wastewater. U.S. Environmental Protection Agency, EPA-600/4-76-049, Cincinnati, Ohio.

U.S. EPA. 1978. Procedure for the Evaluation of Environmental Monitoring Laboratories. U.S. Environmental Protection Agency, EPA-600/4-78-017, Cincinnati, Ohio.

U.S. Geological Survey. 1973. Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples. Techniques for Water Resource Investigations of the USGS, Book 5, Chapter A4. U.S. Government Printing Office, Washington, D.C.

Yates, F. 1937. Design of Analysis of Factorial Experiments. Int. Bur. Soil Sci., Harpenden, England.

SECTION 3

QUALITY ASSURANCE IN BIOLOGICAL RESEARCH

3.1 LABORATORY MANAGEMENT

The character of laboratory management has a strong effect on the quality of work produced. This is true no matter what kind of laboratory it is. For groups of closely related laboratories, such as biological research laboratories, management requirements in various fields of research differ only in detail. After the general aspects of good management have been discussed, the details can be covered field by field.

One of the important concepts of statistical quality control is that the causes of quality problems may be categorized in two ways. One class of causes is that which is within the ability of the individual worker to prevent or correct. The second class is that which is within the capability or authority of management only to handle. Data analysis may be structured so as to assist in separating and identifying these two classes of causes of quality problems.

The first class of causes is called "special" causes; the second class, "common" or "environmental" causes. See Bicking and Deming (1971) for a discussion of the use of this concept in industry. Availability of appropriate data for analysis of this type may not yet be characteristic of most biological research laboratories.

Even before the analysis of data, however, there are certain areas easily identified as being of concern to management. See Table 3.1.1.

3.1.1 On-site Evaluation/Accreditation

The purposes of on-site evaluation include the use of results as a management tool for improving performance of the laboratory and, on the more formal side, for accreditation. Evaluation may be used as a prelude to including a laboratory in a study program or to employing the laboratory on a contract basis. In particular instances requiring evaluation, accreditation by a recognized organization is usually accepted as evidence of the laboratory's capabilities without further evaluation. Accreditation systems include provisions for periodic renewal of the accreditation status.

The evaluation may be conducted by a peer scientist group using more or less formalized check lists, or it may involve a sophisticated rating system. Self-evaluation may be involved, or evaluation by a governmental or independent authority.

TABLE 3.1.1 ELEMENTS OF LABORATORY MANAGEMENT AND QUALITY CONTROL

Management Element	Quality Control
Facilities	• On-site evaluation accreditation
• Building • Services • Equipment	
Personnel	• Certification
• Project director • Project personnel • Support personnel	
Test subjects/materials	• Sampling and testing
Standard procedures	
• Bioassay protocol	• Design review/statistical consultation
• Conduct of experiment	
o Observations/test methods	• Standard test
o Good laboratory practices	• Standard procedures
o Supervision	• Quality policy
o Quality control	• Defined program
• Audit	
Record keeping	• Witnessed log books
Data analysis/reporting	• Statistical treatment

The resources for evaluation available to the biological research community are varied, if not complete. More attention to evaluation has probably been given in clinical laboratories than in nonclinical (animal research) laboratories. While much can be learned from experience in evaluating clinical laboratories the clinical aspects are only a small part of evaluation of biological laboratories in general.

3.1.1.1 General Criteria for Laboratory Evaluation--

General criteria for laboratory evaluation have been promulgated by the government and by standardization agencies. In 1974, OSHA conducted hearings on proposed criteria for laboratory accreditation (OSHA, 1974).

Subsequently the responsibility for developing criteria was transferred from the Labor Department to the Commerce Department, and the National Bureau of Standards developed a plan for centralized administration of laboratory certification. This was published in the Federal Register of February 25, 1976 (Office of the Secretary of Commerce, 1976). It provides for incorporation of existing certification/accreditation programs under a national umbrella and for the establishment of new programs in uncovered areas of science or technology by the professional societies or other organizations. It may eventually include a national certification program for biological laboratories (nonclinical laboratories).

The American National Standards Institute (1971) has adopted laboratory qualification guidelines for use in its certification programs. Also, Committee E-36 of the American Society for Testing and Materials (1977) approved a Standard Practice for General Criteria for Use in Evaluation of Testing and/or Inspection Agencies.

Important evaluation programs in nonclinical laboratories include:

- FDA Good Laboratory Practice Pilot Program, FY77
- American Association for Accreditation of Laboratory Animal Care Procedure
- EPA Procedure for Evaluating Water Bacteriological Laboratories, 1975 (plus state programs)

3.1.1.2 FDA Good Laboratory Practice Pilot Program--

FDA investigations had shown evidence of significant quality control problems in some nonclinical laboratories (FDA, 1976a). Such problems included, but were not limited to:

- poorly conceived, carelessly executed, inaccurately analyzed or reported experiments
- lack of awareness on the part of technical personnel of the importance of protocol adherence
- inaccurate observations, record keeping and record transcription
- failure of management to assure critical review of data or proper supervision of personnel
- use of poorly qualified or poorly trained personnel
- disregard for proper laboratory, animal care, and data management procedures
- failure to monitor studies performed in whole or in part by contract laboratories
- lack of verification of the accuracy and completeness of scientific data
- deliberate falsification of data by management and/or laboratory personnel

These findings led to the issuance of the Compliance Program Guidance Manual (FDA, 1976a) and the Proposed Regulations for Government Laboratory Practice (FDA, 1976b). The Compliance Program directs a pilot effort of inspections of nonclinical laboratories. It is designed to ensure the quality and integrity of the bioresearch data which support the safety of Agency-regulated products.

The program includes completion of a nonclinical Laboratory Inspection Report for each laboratory visited and a Test System Study Report for one or more studies being conducted in the laboratory.

3.1.1.3 Accreditation of Animal Care Laboratories--

Laboratories caring for and using experimental animals may be accredited by the American Association for Accreditation for Laboratory Animal Care (AAALAC). AAALAC uses the Guide for the Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 74-23 (National Research Council, 1972) as its primary reference for determining eligibility for accreditation. These recommendations have been further refined and more rigid or specific standards applied when necessary for carcinogen bioassay in Guidelines for Carcinogen Bioassay in Small Rodents (National Cancer Institute, 1976.) Animal care is discussed in detail in another section.

Facets of the laboratory animal care program which are evaluated by AAALAC include:

- Laboratory Animal Management
    - Housing and care
    - Sanitation practices
    - Feeding, watering, and identification of laboratory animals
    - Provisions for emergency care
- Laboratory Animal Quality and Health
    - Adequate veterinary care
    - Quarantine and isolation of animals
    - Separation by species
    - Diagnosis, treatment, and control of animal diseases
- Personnel
    - Professional personnel
    - Animal care personnel
    - Personal hygiene and personnel health program

- Use of Laboratory Animals
    - Monitoring the use and care of animals
    - Anesthesia and analgesia
    - Surgery and postsurgical care
    - Euthanasia

Institutions seeking to participate in the accreditation program may obtain an application from the AAALAC office at 2317 West Jefferson Street, Joliet, Illinois 60435. A site visit is subsequently made by two representatives of the AAALAC Council on Accreditation. Individuals who have extensive expertise and experience in laboratory animal science are selected as consultants for the accreditation program. The site visitors make a complete and thorough review of all aspects of the animal care program carried out at the institution being evaluated. A detailed report is submitted to the Council on Accreditation, and after thorough review, recommendations of the Council are forwarded to the Board of Trustees for action. Following this, a detailed report is sent to the applicant institution outlining the decision taken and providing a detailed analysis of the program. Every effort is made to provide a thorough and comprehensive review of all programs under evaluation. In essence, the entire program closely follows the review processes which have been developed by granting agencies for evaluating the merits of grant applications.

Through the accreditation program, institutions have been able to document their deficiencies and respond to them. These deficiencies vary, but in 1973 an analysis of the deficiencies encountered in the AAALAC program which were serious enough to warrant not accrediting the institution was made. Examples of deficiencies listed in the order of prevalence are:

Improper sanitization
Caging of insufficient size or design
Improper quarantine and isolation program
Improper environmental control
Improper sanitary waste disposal
Personnel deficiencies
Inadequate physical plant conditions
Inadequate control of animal diseases
Inadequate personnel health program
Feeding and watering deficiencies
Inadequate emergency procedures
Inadequate animal surgery and postsurgical care program
Inadequate storage space
Inadequate vermin control program
Overcrowding of animals
Administrative problems
Inadequate illumination
Inadequate identification procedures
Inadequate euthanasia practices

Approximately 70% of the institutions that did not gain accreditation after the first site visit ultimately improved their animal care program to an accreditable level.

3.1.1.4 Evaluation of Water Bacteriological Laboratories--

The Municipal Environmental Research Laboratory of EPA, at Cincinnati, has published a Handbook for Evaluating Water Bacteriological Laboratories

(U.S. Environmental Protection Agency, 1975). Here again, many of the sections are applicable to many kinds of laboratories. Of particular interest are the guidelines, or check lists, which accompany each chapter.

An example, the Guidelines on Laboratory Management, is reproduced in Figure 3.1.1.

Laboratory Records

Results assembled and available for inspection ________

Data processed rapidly through laboratory and engineering sections ________

Adequate data retention, efficient filing system, and prompt channeling of report copies ________

Number of tests per year

MPN Test - Type of sample________________________

Confirmed (+)__________(-)__________(Total)__________

Completed (+)__________(-)__________(Total)__________

MF Test - Type of Sample________________________

Direct Count (+)__________(-)__________(Total)__________

Verified Count (+)__________(-)__________(Total)__________

Personnel

Adequately trained or supervised for bacteriological examination of water________________________

Personnel involved:

Professional staff (total)________________________

Sub-professional support (total)________________________

Clerical assistance (total)________________________

Reference Material

Copy of Standard Methods (current edition) available in the laboratory ________

State or Federal manuals on bacteriological procedures available for staff use ________

Scientific journals in water research accessible ________

Laboratory Facilities

Laboratory room spaced and bench-top area adequate for needs during peak work periods ________

Prep room space adequate and located near laboratory ________

Sufficient cabinet space for media, chemicals, glassware, and equipment storage ________

Facilities clean, with adequate lighting and ventilation, and reasonably free from dust and drafts ________

(Continued)

Figure 3.1.1 Guidelines on Laboratory Management.

Office space and equipment available for processing water examination reports and mailing sample bottles ________

Laboratory Safety

Personnel and carts permitted mobility without obstructions that cause accidents ________
Adequately functioning autoclaves and stills, with periodic inspection and maintenance ________
Electrical service conforms to local, state or National Electrical Codes ________
All electrical equipment grounded through three-wire system or separate ground to cold water pipe ________
Foam-type and carbon dioxide fire extinguishers accessible ________
Fire exits from laboratory clear at all times ________
Emergency (deluge) shower accessible and functional ________
Safety features such as pipet waste jars with disinfectant, centrifuge shield, splatter guard, and blender covers employed to avoid bacterial aerosols ________
Approved practices for handling and disposing of radioactive chemicals used in special bacteriological procedures ________
First aid supplies available and not out-dated ________
Personnel trained to safely handle steam, flames, chemicals, pathogens, etc. ________
Personnel indoctrinated in first aid emergency procedures, fire control, etc. ________
Broken glass, sharp needles, etc., properly handled and disposed of ________

Figure 3.1.1 Continued .

Other sections of the Handbook contain guidelines on specific laboratory activities, as follows:

- Sampling and monitoring response
- Laboratory apparatus
- Glassware, metal utensils and plastic items
- Laboratory materials preparation
- Culture media specifications
- Multiple tube coliform procedures
- Membrane filter coliform procedures
- Supplementary bacteriological methods
- Reports

A number of states conduct laboratory certification programs:

- Connecticut State Department of Health, Laboratory Standards Section, approves water laboratories
- New York State Department of Health, Division of Laboratories and Research Programs, approves

laboratories analyzing potable water

- North Carolina State Department of Natural and Economic Resources certifies air and water analytical laboratories
- Oklahoma State Water Resources Board certifies water and wastewater laboratories.
- California State Department of Health, Water Resources Board licenses water laboratories

3.1.1.5 Accreditation of Industrial Hygiene Laboratories--

The American Industrial Hygiene Association in association with the Health Physics Society accredits laboratories based on criteria under the following headings:

- Laboratory direction
- Laboratory supervision
- Laboratory personnel
- Proficiency testing
- Quality control and equipment
- Facilities
- Records

The proficiency testing is carried out by NIOSH under their PAT (Proficiency Analytical Testing) Program. Satisfactory performance is based on a statistical estimation of whether the results obtained are probably representative of analytical competence.

Quality Control procedures considered essential include:

- Routinely introduced samples of known content along with samples submitted for analysis
- Routine checking, calibrating and maintaining adequate performance of equipment and instruments
- Routine checking of procedures and reagents
- Good housekeeping, cleanliness, and general orderliness of premises

3.1.1.6 Programs of Clinical Laboratories--

In the clinical laboratory area, The College of American Pathologists conducts a Laboratory Inspection and Accreditation program that has many interesting aspects. Each laboratory seeking accreditation must be enrolled in the CAP Proficiency Testing Program (Surveys). Accreditation is renewable every two years. A computer-processed check list is provided for self-evaluation in the interim year. Other services offered by CAP include a Quality Assurance Service, computerized tabulations, plots, and analyses of a laboratory's daily quality control data; a Proficiency Evaluation Program (PEP) with self-evaluation testing kits for physicians; and a Product Evaluation Program for suppliers of laboratory products.

The Center for Disease Control conducts a laboratory licensing program under the Public Health Service Act as amended in 42 USC 20 et seq. Participation in a proficiency testing program is required of all laboratories covered under the act. See Center for Disease Control, 1975 for a description of the proficiency testing program. All laboratories having acceptable results in the program are classed as "licensed" laboratories. The following areas of testing are covered:

- Microbiology and serology
- Clinical chemistry
- Hematology
- Immunohematology
- Radiobioassay

In December 1973, the Technical Analysis Division of the Institute for Applied Technology at the National Bureau of Standards published a study of results of the CDC Proficiency Testing Program (National Bureau of Standards, 1973). This is an interesting assessment of the value of proficiency testing.

3.1.1.7 Significance of On-site Evaluations--

Acceptable ratings as a result of on-site evaluation usually infer capability of the laboratory for doing a satisfactory job. The rating itself, or even resulting certification or accreditation, does not necessarily mean that performance by the laboratory will be everything that could be desired. That is the reason why most programs for laboratory evaluation add a requirement for testing of split samples (proficiency testing). Successful identification of samples in a collaborative proficiency testing system increases the confidence that can be placed in a laboratory's work.

Some of the evaluation systems described are administration rather than operation oriented. For example, the FDA system is keyed to the Proposed Regulations for Good Laboratory Practices. Although casually referred to as GLP's, these are regulations only and do not contain explicit procedures for conduct of experiments or making of observations or tests. The NCI Guidelines (NCI, 1976) on which most animal research laboratory evaluation is based are looked upon properly as guidelines and not standards. The EPA procedures for monitoring laboratories and for water bacteriological laboratories are much more explicit as to equipment requirements, test methods, and operating procedures. Before the quality of a laboratory's results can be improved, much more direction must be given to it in the form of material specifications, standard test methods, good techniques of experiment design, standard operating procedures and quality control techniques. It is identification of and characterization of the effectiveness of such specific operating procedures that really makes on-site evaluation significant in improving laboratory operations.

3.1.2 Laboratory Personnel

The study director and principal personnel to be associated with a study should be identified prior to the start. This provides an opportunity for review of appropriateness of the staff. Obviously, the study director should be an expert in the area of the study. He, or other personnel in supervisory positions should be well qualified by academic training and experience. The specific qualifications should be spelled out in job descriptions. Where experience is lacking, in-house training, perhaps on a continuous basis, is desirable.

For some disciplines involved in biological research, certification programs are available. For example, veterinarians who are needed to conduct broad-based laboratory animal preventive medicine programs are certified by the American College of Laboratory Animal Medicine. This program would include screening representative numbers of animals, microbiologically and virologically, gross and microscopic evaluation of necropsy specimens, and other tests. Depending upon the animal species, one or more of the above should be accomplished as often as necessary to ensure that only animals of the required quality are placed on experiments.

Quality assurance also includes staffing animal care facilities with properly trained personnel. The American Association for Laboratory Animal Science has established national testing standards and there are three skill levels currently recognized. Training programs may be geared to certifying technicians under this program.

3.1.3 Biological Sampling and Testing

3.1.3.1 Biological Tests

Some of the common techniques of analysis in biological research, with particular reference to the water environment, are given in Table 3.1.2.

These techniques are described briefly as follows:

- Count and identification - A useful test to determine overall the health of species in an ecosystem by providing data on standing crop and community structure
- Weight/length - The growth rate of a community is determined and compared to previous studies to indicate a change in environmental quality
- Flesh tainting - A test of palatability to determine if sublethal chemical doses have imparted an unpleasant taste to fish or shellfish flesh
- Acetylcholinesterase - An indirect test of the previous effect of organophosphate pesticides on the central nervous system of fish in a water system
- Tissue analysis - A qualitative or quantitative test of concentration of histological effects of various materials including metals and pesticides in flesh
- Stomach contents - An analysis of this will indicate the type and amount of feeding done by an organism prior to collection
- Wet, dry and ashfree weight - These tests are used to make quantitative tests of the standing crop of a population

TABLE 3.1.2 PARAMETERS OF BIOLOGICAL COMMUNITIES
MOST COMMONLY ANALYZED (U.S. EPA, 1976)

Community	Parameter	Units
Plankton	Counts	Numbers/ml by genus and/or species
	Chlorophyll a	mg/m^3
	Biomass (ash-free, dry weight)	mg/m^3
Periphyton	Counts	Number/mm^2
	Chlorophyll a	mg/m^2
	Biomass (ash-free weight)	mg/m^2
	Autotrophic index	$\frac{\text{Ash-free weight (mg/m}^2\text{)}}{\text{Chlorophyll a (mg/m}^2\text{)}}$
Macrophyton	Areal coverage	Maps by species and species associations
	Biomass (ash-free weight)	g/m^2
Macroinvertebrate	Counts	Grab - number/m^2 Substrate - number/sampler
	Biomass	g/m^2
	Toxic substances	mg/kg
Fish	Toxic substances	mg/kg
	Counts	Number/unit of effort, expressed as per shocker hour or per 100 feet of a 24-hour net set
	Biomass (wet weight)	Same as counts
	Condition	$K(TL) = \frac{10^5 \text{ x weight in grams}}{L^3 \text{ (length in mm)}}$

- Chlorophyll a - An estimate of the algal biomass is obtained which roughly indicates the standing crop
- ATP determinations - ATP tests measure the total viable plankton biomass
- Diatom species proportional count - This test indicates the health of a diatom community by comparing the results through the use of a diversity index

3.1.3.2 Sample Preservation and Handling--

Sample preservation is distinctive for each area of biological research and for each parameter to be measured. When a chemical preservative is used, extreme agitation may be necessary to disperse the chemical preservative throughout the sample. If the preservative cannot be dispersed, refrigeration or freezing may be an appropriate alternative.

Various preservatives exist to maintain species in the desired condition. Advantages and disadvantages of various preservatives are given in Table 3.1.3.

Preservation and handling procedures are given in Table 3.1.4 for:

- Benthic Macroinvertebrates
- Fish
- Macrophytes and Macroalgae
- Periphyton
- Periplankton
- Zooplankton

3.1.4 Preparation of Study Protocols

A general outline for a bioassay protocol is as follows:

- Purpose of study
- Design of experiment
- Conduct of experiment
- Observations and tests
- Records and reports

3.1.4.1 Purpose of Study--

There should be a brief, direct statement of the purposes of the study. For example, the purpose of a chronic feeding study using rats might be:

- Effects of test material on the reproduction process in rats

  o Fertility
  o Maintenance of offspring
  c Postpartum effect
  o Weaning period

- Chronic toxicity of test materials

- Carcinogenesis due to exposure during organogenesis, fetal development, location, and throughout life.

TABLE 3.1.3 COMPARISON OF CHEMICAL PRESERVATIVES FOR BIOLOGICAL PARAMETERS (SLACK ET AL., 1973; U.S. EPA, 1976)

Chemical	Advantage	Disadvantage
General Preservation		
1. Formalin (5-10% formaldehyde)	Kills species; infinite holding period	Objectionable odor, can cause contraction or deflaggelation
2. 70% ethanol		Needs neutralization w/sodium tetraborate
3. 40% isopropanol	Safer and easier for analyst to use; same advantages as formalin	Can cause contractual reaction
4. Oxyquinoline (2% solution)(8-hydroxyquinoline sulfate)	Safer and easier for analyst to use; can be added as solid in premeasured packets; same advantages as formalin	Can cause contractual reaction
5. Merthiolate solution	Morphology and color of algae are retained; distinguish between zoo- and phytoplankton	Does not produce a sterile sample
6. Glycerin (added with 1, 2 or 3)	Prevents tissues from drying	
7. Copper sulfate	Retains bluegreen color of algae	Stains other material; also toxic
8. Detergent	Lowers surface tension to prevent clumping or clinging to container walls	
Stains		
9. Lugols's solution	Stains algae; aids settling by releasing gases	Samples stable only one year

TABLE 3.1.4 RECOMMENDED PRESERVATION AND HANDLING METHODS (U.S. EPA, 1976)

Item	Preservation Method	Holding Time	Container
BENTHIC MACROINVERTEBRATES			
Count/identification	70% ethyl alcohol	1 year	Glass or plastic
Wet and dry weight	Refrigerate at 4°C or ice	Immediate to 24 hours	Glass or plastic
Ash-free weight	Filter and refrigerate at 4°C	6 months	Glass or plastic
Calorimetry	Refrigerate at 4°C or ice. Once filtered, store in desiccator	Immediate to 24 hours	Glass or plastic
Radio-tracer studies	Freeze	1 year	Glass or plastic
Flesh tainting	Freeze	Indefinite	Glass or plastic
Tissue analysis	Freeze	Indefinite	Glass or Plastic
FISH			
Count/identification	10% Formalin, add 3 g borax and 50 ml glycerin per liter*	Indefinite (1 year; sooner is better)	Borosilicate glass or polyethylene
Weight/length	None - analyze immediately	None	None
Flesh tainting	Clean, then freeze	Indefinite	Borosilicate glass or polyethylene Aluminum foil
Acetylcholinesterase	Freeze	Indefinite	Aluminum foil

(continued)

TABLE 3.1.4 (Continued)

Item	Preservation Method	Holding Time	Container
Tissue analysis	Freeze	Indefinite	Borosilicate glass or polyethylene Aluminum foil
Stomach contents	Remove stomach from fish and preserve in 10% Formalin (as for count/identification)	Indefinite (1 year, prefer sooner)	Glass or plastic
MACROPHYTES AND MACROALGAE			
Count/identification	5% Formalin	1 year	Glass or plastic
Wet and dry weight	Refrigerate at 4°C or ice	Immediate to 24 hours	Glass or plastic
Ash-free weight	Freeze	6 months	Glass or plastic
Chlorophyll a	Freeze at -20°C	1 month (keep out of light; acid)	Glass or plastic
PERIPHYTON			
Count/identification	5% neutral Formalin	6 months	Opaque glass or plastic
Diatom species proportional count	5% neutral Formalin	6 months to indefinite	Glass or plastic
Wet and dry weight	Refrigerate at 4°C or ice (do not freeze)	Immediate to 24 hours	Glass or plastic
Ash-free weight	Freeze at -20°C	6 months	Glass or plastic

(continued)

TABLE 3.1.4 (Continued)

Item	Preservation Method	Holding Time	Container
Chlorophyll determination	Immediate extraction in 90% aqueous acetone; store at -20°C	1 month (keep out of light and acid)	Glass or plastic
ATP determination	Extract by boiling with Tris Buffer; store extract at -20°C	6 months	Glass or plastic
PHYTOPLANKTON			
Count/identification	a. 5% neutral Formalin b. Merthiolate	a. Indefinite b. 1 year	Opaque glass or plastic
Wet and dry weight	Refrigerate at 4°C or ice (do not freeze)	Immediate to 24 hours	Glass or plastic
Ash-free weight	Filter and freeze at -20°C	6 months	Glass or plastic
Chlorophyll a	Extract immediately or filter and freeze in desiccator at -20°C	1 month (keep out of light and acid)	Glass or plastic
Diatom species proportional count	5% Formalin	6 months to indefinite	Opaque glass or plastic
Calorimetry	Refrigerate at 4°C or ice; once filtered, store in desiccator	Immediate to 24 hours	Glass or plastic
ATP determination	Extract by boiling with Tris Buffer, freeze extract at -20°C	6 months	Glass or plastic

(continued)

TABLE 3.1.4 (Continued)

Item	Preservation Method	Holding Time	Container
ZOOPLANKTON			
Count/identification	5% Formalin or Lugol's solution plus 50% glycerin, or 70% ethanol plus 50% glycerin	1 year	Glass or plastic
Wet and dry weight	Refrigerate at 4°C or ice (do not freeze)	Immediate to 24 hours	Glass or plastic
Calorimetry	Refrigerate at 4°C or ice (do not freeze); once filtered, store in desiccator	Immediate to 24 hours	Glass or plastic
ATP determination	Immediately extract by boiling with Tris Buffer; store extract at -20°C	6 months	Glass or plastic

* Replace solution with alcohol after 1 week.

3.1.4.2 Design of Experiment--

This section should contain:

- Identification of the biological subject
- Identification of the test material
- Route of administration of test material
- A table giving groups, group sizes and dose levels
- Information on how dose levels were selected
- Exposure schedules and duration of test
- Special instructions for administration of doses
- Description of controls
- Description of special test equipment
- Special instructions necessary to complete the plan of work

The test subject should have been selected with all the criteria for appropriate test species in mind. The test material will have been selected for some particular purpose or with accepted rules for prioritization in mind.

The animal species, the nature of the test material, the milieu of the experiment and the purposes of the experiment all have a bearing on the route of administration of the test material. A feeding study, for example, usually implies incorporation of the test material in the diet. A problem arises if the material is highly volatile or is a gas. It is unlikely that large quantities of these materials would remain incorporated in the feed and be ingested. It is possible to administer gases orally through use of a carrier, such as water or corn oil, in which the material is soluble, and to incorporate that into the diet. Alternatively, microencapsulation could be used, if a nontoxic material through which the gas is not diffusable could be found. Intubation, or gavage, of the gas and the carrier is also a possibility. As a quality control procedure, fresh solutions would have to be prepared frequently in intubation studies.

Soluble materials can be administered in the drinking water. If volatile, a closed system of glass and stainless steel is required and rubber washers, etc., must be avoided. Analytical chemical analysis will be required to verify that the stock solutions are fresh. To ensure integrity of the closed system, water should be supplied from glass bottles with plastic screw caps fitted with stainless steel siphon tubes containing stainless steel balls.

Inhalation routes, skin or eye applications, aquatic experiments, in the laboratory or in the field, plant experiments, and so on, all require careful description of the route of administration in the Design of Experiment Section. Procedures for quality control of administration of the test substance should be included in the Conduct of Experiment section of every protocol. More detail will be found in Sections dealing with different kinds of bioassays.

The table of groups, group sizes and dosage levels may take the form shown below for a chronic mouse feeding study:

Group No.	No. of Animals Male	 Female	Dose Levels*
1	50	50	Control
2	50	50	Low (1/8 MTD)
3	50	50	Medium low (1/4 MTD)
4	50	50	Medium (1/2 MTD)
5	50	50	Medium high (3/4 MTD)
6	50	50	High (MTD)

* Active material

- Control - no-treatment diet
- Maximum tolerated dose (MTD) calculated from available subacute or subchronic data
- Test material will be incorporated in the diet over a 24-month period
- Feed and water will be offered ad libitum

The number of groups, the number of animals per group, the scale selected for dose levels and the proposed method of data analysis are all proper subjects for review by a statistician at the Experiment Design stage. As a matter of good practice, it is desirable to have the statistician's signature on the protocol to indicate that the design is adequate.

The statistical design of experiments has been described in Section 2.2 of the Quality Assurance Guidelines. As an illustration of the criticality of number of animals per group, the following tabulation gives an example of how number is influenced by the expected frequency of finding of an effect in the control group. The number of animals per group required to show a 15% difference between the control and a treated group with 95% probability is:

Percent Animals Affected Control	 Treatment Group	Group Size Required
0	15	36
10	25	66
20	35	85

Note that the statistician's interest is in the magnitude of the difference it is desired to detect, the acceptable level of significance of that difference, and sufficient history of the use of the assay procedure (including the particular test subject) to determine the expected frequency of the occurrence of an effect in the control.

Quality control over the design of experiment processes is exercised through a review which may be by a peer committee from the study director's lab or it may involve outside consultation. The approval system should include signing of the written protocol before start of work by the study director and the head of the laboratory, and by a statistician.

3.1.4.3 Conduct of Experiment--

In this section of the protocol, each procedure for conduct of experiment should be spelled out in sufficient detail that there can be no mistake regarding the details of day-to-day operations of the laboratory. In these Quality Assurance Guidelines, we recommend that the operational steps and the quality control activities be laid out in parallel columns or otherwise highlighted in association with each other. The first example given here illustrates the parallel column arrangement. The other examples illustrate a different, and more space-saving format.

EXAMPLE 1: CHRONIC MOUSE FEEDING STUDY

Quarantine

- Quarantine all animals upon receipt.
  QUALITY CONTROL -- Hold in quarantine for 1 week.

Animal Identification, Randomization, and Housing

- Assign to study group following quarantine.
  QUALITY CONTROL -- Use randomization procedure (see Section 2.7)

- Prior to study initiation, all animals will be weighed and appropriate adjustments made to achieve an equivalent mean body weight value between the groups.

- Identify by cage, group, and individually.
  QUALITY CONTROL -- Use ear tags and durable cage markers.

- All animals will be housed by sex and dosage, five per cage.
  QUALITY CONTROL -- Follow NCI Guidelines for cage space per animal.

Test and Control Materials

QUALITY CONTROL -- Conduct stability tests; return samples to sponsor for analysis, if requested.

Feed

- The basic diet will consist of a commercial rodent ration.

- The diet will be analyzed for the parameters listed below:
  - Polychlorinated biphenyls and chlorinated hydrocarbons
  - Antibiotics
  - Lead, arsenic, mercury
  - Estrogen
  - Aflatoxins
  - Nutritional content

  QUALITY CONTROL -- The frequency of these analyses is to be determined by the sponsor.

- The test material will be incorporated into the basal diet on a weight/weight basis and thoroughly mixed in a twin-shell blender to provide the appropriate diet level for each group.
  QUALITY CONTROL -- The uniformity and concentration of the test material in the feed will be demonstrated prior to administration.

- Fresh batches of the diet will be prepared weekly.
  QUALITY CONTROL -- Samples will be taken and tested from each batch of feed.

Water

- Offer *ad libitum*.
  QUALITY CONTROL -- Sample on a quarterly basis and analyze for heavy metals and coliforms.

EXAMPLE 2. PRIMAL DERMAL IRRITATION STUDY IN RATS

(Illustration of special procedures only)

Preparation of Treatment

- The hair will be clipped from the backs, and on one side a 1-inch square will be abraded by making minor incisions through the stratum corneum, but not deep enough to disturb the derma (that is, not sufficiently deep to produce bleeding).
  QUALITY CONTROL -- Observe for bleeding.

- Treatment will be applied with animals immobilized in an animal holder. The entire trunk will be wrapped with a rubber dam or Saran wrap for 24 hours.
  QUALITY CONTROL -- Follow plan in design of experiment.

EXAMPLE 3. CHRONIC INHALATION STUDY IN RATS

(Illustration of special procedures only)

Generation of Atmospheres

- Generate atmospheres by method appropriate to the test material. For volatile liquids, generate high concentrations by passing compressed air through the liquids at constant rates. Reduce to dilution with filtered warm air drawn through the chambers which operate under negative pressure. QUALITY CONTROL -- Monitor continuously prior to exposure of animals. Calibrate the monitoring equipment (such as hydrocarbon analyzer) with the substance being tested. The range of calibration points will encompass the selected dosage levels.

QUALITY CONTROL -- Aliquots of the test substance will be introduced into large gas sampling bottles of known volume. After vapor concentration reaches equilibrium the aliquot will be introduced into the analyzer.

- Analyzer should be equipped with 10-point automatically timed solenoid system: 1-8, Level of substance in eight chambers; 9, Room atmosphere; 10, Combined stack effluent.
  QUALITY CONTROL -- Sample four times each day for 10 minutes per sampling point. Adjust flow as required.

EXAMPLE 4. IN VITRO TRANSFORMATION OF BALB/3T3 CELLS

Seeding

QUALITY CONTROL -- The vehicle for the test chemical is used in the negative control plates.

- Approximately 10,000 cells are seeded into a 60-mm plastic plate and incubated 24 hours to firmly attach the cells. This plate will be used to assess transformation. Simultaneously with seeding, separate plates will be seeded at 200 cells per plate to obtain toxicity determinations.

Dosing

- The positive control and four doses of test chemicals are added to the transformation and toxicity plates. Treatment with the test chemicals will consist of exposing the cells in an airtight enclosed chamber to either vapors or a gaseous state of the test materials. Various dose levels will be achieved by varying the length of exposure to a fixed level of the vapors or gas. Treatment will be terminated by removing the plates from the chamber and replacing the media with fresh growth media.

Incubation

- Following treatment, the cells will be incubated for 3 to 4 weeks before they are scored for transformed foci. The toxicity plates will be scored after only 1 week. During the incubation periods, growth media will be changed twice weekly.

3.1.4.4 Observations and Tests--

All observations and tests required should be described fully in this section of the protocol. Quality control procedures should be identified. Detail will vary sharply from one type of bioassay to another. Typical examples are given below.

EXAMPLE 1. TERATOGENICITY STUDY IN RABBITS

Observations and Tests

- Weekly records will be made on individual female parents with respect to body weight (day 0, 6, 12, 18, 29), appearance, behavior, and survival.
- At termination, brain, liver, and kidney weights, and the calculation of liver/brain weight ratios will be done on all adult females in each group. The following observations will be recorded on does killed at termination and on their progeny:
    - Number and placement of uterine sites
    - Number and placement of live, dead, and resorbed fetuses
    - Number of corpora lutea
    - Fetal weight and length (crown to rump)
    - External fetal anatomy
    - Any gross abnormalities
    - Gross necropsy evaluation on all fetuses, pups, and does.
- All fetuses and pups will be cleared and stained with Alizarin Red S for evaluation of skeletal effect. The reproductive organs of the female parents will be preserved in 10% neutral formalin and held for possible future histologic evaluation.

  QUALITY CONTROL -- All data will be evaluated statistically.

EXAMPLE 2. PRIMARY DERMAL IRRITATION STUDY IN RABBITS

Observations and Tests

- After 24 hours of exposure, the patches will be removed and the resulting reactions will be evaluated on the basis of scores indicated in the following table:

EVALUATION OF SKIN REACTIONS

I. Erythema and Eschar Formation

0	No erythema
1	Very slight erythema (barely perceptible)
2	Well-defined erythema
3	Moderate to severe erythema
4	Severe erythema (beet redness) to slight eschar formation (injuries in depth)
4	Total possible erythema score

II. Edema Formation

0	No edema
1	Very slight edema (barely perceptible)
2	Slight edema (edges of area well defined by definite raising)
3	Moderate edema (raised approximately 1 mm)
4	Severe edema (raised more than 1 mm and extending beyond area of exposure)
4	Total possible edema score

- Readings will be made again at the end of 72 hours. The reading on each rabbit will be recorded.

EXAMPLE 3. PATHOLOGICAL PROCEDURES

In chronic studies, whole-animal test pathological procedures are required and these can be standard. A proposed standard for pathological procedures is as follows:

Personnel

- A board-certified veterinary pathologist with experience in laboratory animal pathology will be responsible for all pathology procedures, evaluations, and reporting. Histology technician(s) will be supervised by an HT/ASCP certified technician. Personnel trained and experienced in laboratory animal dissection to recognize gross abnormalities will be prosectors. Qualified personnel will be available for weekend coverage to necropsy dead or moribund animals, or to refrigerate them for necropsy at the earliest possible time.

Facilities

- Refrigeration is available for holding dead animals until necropsy. Animals will not be frozen. The histology laboratory is separated from the necropsy area and is equipped with automatic tissue processors, microtomes, embedding and stirring equipment, and supplies.

- Adequate storage facilities are available to store and file histologic slides, tissue blocks, and wet tissues for the duration of the contract. This facility is vermin proof and temperature controlled. The area provided for trimming of fixed tissues has adequate ventilation and exhaust hoods for removal of formaldehyde fumes.

Gross Necropsy

- A blood smear will be taken from all animals at the time of necropsy. Whether this smear ultimately will be read or not will depend on the observations made during gross necropsy or histopathologic review. Therefore, all smears will be fixed and retained for possible future use.

A complete gross necropsy is defined as an examination and harvest of all of the following tissues:

Gross lesions and tissue masses (and regional lymph nodes, if possible)
Skin
Mandibular lymph node
Mammary gland
Salivary gland
Thigh muscle
Sciatic nerve
Sternebrae, vertebrae or femur, including marrow
Costochondrial junction, rib
Thymus
Larynx
Trachea
Lungs and bronchi
Heart
Thyroid
Parathyroids
Esophagus
Duodenum
Jejunum
Ileum
Colon
Cecum
Rectum
Mesenteric lymph node
Liver
Pancreas
Spleen
Kidneys
Adrenal glands
Bladder
Seminal vesicles
Prostate
Testes
Ovaries
Uterus
Nasal cavity *
Brain
Pituitary
Spinal cord
Eyes *

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* Always examined; harvested only when lesions are present.

- All animals that die or are killed will receive a complete gross necropsy (unless cannibalism or autolysis preclude all or part of the examination).

- The gross dissection and evaluation will be performed by or under the direct supervision of the pathologist(s).

- Peripheral blood smears from the heart, tail, or toe will be prepared for animals in those cases where neoplasia of the lymphoid system or of the bone marrow is suspected (as evidenced by an enlarged spleen, liver or lymph node, or by a watery appearance to the blood, indicating an anemic condition). Smears will be air-dried, then fixed in absolute methanol within 24 hours. Touch preparations will be prepared from any enlarged spleen. If lymphoid organs other than the spleen are enlarged, then a touch preparation will be made from those affected organs. Smears will not be stained unless requested by the pathologist.

- All tissues and/or organs will be examined in situ, then dissected from the carcass, re-examined and fixed in 10% neutral buffered Formalin. Lungs of mice and rats will be fixed in their entirety after opening and examining the trachea and main-stem bronchi. The calvarium will be removed and the dorsal nasal bone removed for examination of nasal turbinates. The entire skull will be fixed with the brain in situ. Other tissues will be fixed at a thickness not exceeding 0.5 cm.

- Urinary bladders will be opened and examined before fixation. Contracted, empty bladders may be partially distended with Formalin before opening to reveal small lesions that may be obscured by epithelial folds. One kidney will be longitudinally bisected, the other kidney will be transected and examined before fixation.

- The entire mucosal surfaces of the esophagus, stomach, small and large intestine and rectum will be opened and examined before fixation. If the gastrointestinal tract can be visualized, it will not be opened.

- Representative portion(s) of large tissue masses (including surrounding unaffected tissues) will be fixed. Small masses (including surrounding unaffected tissues) will be fixed. Very small masses will be fixed in their entirety. All gross lesions will be recorded in narrative, descriptive terms to include location, size, number, shape, color and texture. Several thoracolumbar vertebrae will be fixed with the spinal cord _in situ_. Carcasses of animals will be discarded following necropsy.

Trimming of Fixed Tissue

- Tissue fixation time will be no less than 48 hours or no more than 12 weeks. Tissue trimming will be performed by or under the supervision of the pathologist(s) with the gross necropsy descriptions available. Tissues will be trimmed to a maximal thickness of 0.3 cm for processing.

- Multiple portions of tumors or masses will be submitted if large or variable in appearance, and surrounding normal tissue will be included. Parenchymal organs, e.g., liver, will be trimmed to allow the largest surface area possible for examination. One longitudinal and one transverse section through the entire cortex and medulla of each kidney will be submitted. Entire coronal sections of both right and left lungs, including main-stem bronchi, will be submitted for mice and rats; the portion best representing the lesion or a portion thereof will also be taken. A parasagittal section of brain will include: (a) frontal cortex and basal ganglia, (b) parietal cortex and thalamus, and (c) cerebellum and pons.

- Hollow organs will be trimmed and blocked to allow a cross-section slide from mucosa to serosa. Small (less than 0.3 cm) endocrine organs, lymph nodes and tissue masses will be submitted intact.

Histologic Technique

- Paraffin sections will be cut at 4-6 micrometers and stained with hematoxylin and eosin. Blood smears will be air dried at necropsy, then fixed in absolute methanol for 5 minutes. Smears will be stained with Wright's, Giemsa or Romanovsky stain when requested.

Histopathologic Examinations

- Histopathologic examinations will be performed on: 1) all vehicle or negative control animals in the chronic study; and 2) all test group animals in the chronic study.

Complete histopathologic examination is defined as examination of the following:

Gross lesions and tissue masses (and regional lymph nodes, if possible)
Blood smear (as required by the pathologist)
Mandibular lymph node
Mammary gland
Salivary gland
Sternebrae, femur or vertebrae, including marrow
Thyroid
Parathyroid
Esophagus
Stomach
Small intestine (one section)
Colon
Liver
Gallbladder
Prostate
Seminal vesicles
Testes
Ovaries
Uterus
Brain (three sections including frontal cortex and basal ganglia cortex and thalamus; and cerebellum and pons)
Thymus
Trachea
Lungs and main-stem bronchi
Heart
Pancreas
Spleen
Kidneys
Adrenal glands
Urinary bladder
Pituitary
Spinal cord (if neurological signs are present)
Eyes (if grossly abnormal)

- Tissues will be blocked in a systematic manner to enhance efficiency in histopathologic examinations. All pathologic diagnoses will be made or confirmed by the pathologist(s).

Submission of Pathology Results (Individual Animal Data Record Form)

- Histopathologic diagnoses of all lesions will be entered under Organ and Diagnosis. Primary versus metastatic tumors, e.g., liver hepatocellular carcinoma; and lung, hepatocellular carcinoma (metastatic) will be indicated.

- Descriptive narratives of gross necropsy findings will be provided for all animals. The number as well as description of tissue masses will be included. If they are confluent or too numerous to count (TNTC), this will be noted.

Residual Material

- All blocks, wet tissues, and slides of chronic animals (test, vehicle control, and untreated control) will be retained in a vermin-proof, temperature-controlled area until termination of the bioassay investigation. At completion of the program, these residual materials will be organized, packed, marked, and shipped to the sponsor. Clearance to ship will be requested before any action is taken to ship.

- Wet tissues (residue from harvested tissues, not carcasses) will be stored in two sealed plastic bags one inside the other and organized by histology numbers. A permanent ink label will be placed between the two bags showing the name of the contractor and the histology number. Once the bags are organized, they will be packed in 350 lb-test double-wall cardboard boxes and marked on one end to show:

Name of contractor

Contract number

Chemical number

Animal group number(s)

Histology numbers in that box.

- These boxes will be sealed shut with shipping tape, bound with filament tape, and shipped to the sponsor upon receipt of clearance to do so.

- Blocks will be resealed with paraffin, organized by histology number, and labeled or permanently marked with the name of the contractor and the histology number. When histopathology is complete and the residual material is to be prepared for shipment to the sponsor, blocks will be placed into single-wall cardboard boxes the size of approximately 80 blocks and then these smaller boxes placed into 350 lb-test double-wall cardboard containers approximately 16" x 18" x 7-1/2". Boxes will be marked on one end to show the information indicated in the above. Shipping cartons will be sealed with pressure tape and bound with filament tape for shipment.

- Slides will be organized by histology number. They will be placed in metal slide cabinets. Each metal slide cabinet will be marked to show the range of histology numbers and the name of the contractor. These cabinets will contain a list identifying the name of the contractor, the number of slides, and the cross-reference information,i.e., animal numbers, histology numbers, and chemical numbers, which will allow complete identification of the contents.

- A master log of histology number assignments will be provided to the sponsor along with the first shipment of slides. Since this log may not be complete when the first shipment of slides is made, updated versions of the log will be provided.

Pathologic Material to be Retained by the Contractor Until Termination and Final Reporting of Study

- All wet tissues will be stored in plastic bags, sealed, clearly and permanently labeled and retained in a vermin-proof area. All histologic slides and paraffin blocks will be sealed with paraffin.

- The grouping of mouse tissues on the microslides will be as follows:

Slide 1: Brain (2); Pituitary: Thyroid/Parathyroid/Trachea/Esophagus

Slide 2: Heart; Kidney (2); Adrenal glands (2); Liver with gallbladder (2); Thymus; Spleen; Pancreas

Slide 3: Lung and main-stem bronchi

Slide 4: Stomach; Small intestine (2); Large intestine (2); Urinary bladder

Slide 5: Testes/Epididymis/Seminal vesicles (2)/Ovary (2); Prostate/Uterus; Salivary gland with mandibular lymph node; Mammary gland; Skin

Slide 6: Bone; Bone marrow; Spinal cord (if neurological signs are present)

Slide 7: Tissue masses (suspect tumors)

Slide 9: Multiple sections of skin

Slide 10: Blood smear (or eye, if abnormal)

3.1.5 References

American National Standards Institute. 1971. Laboratory Qualification Guidelines for ANSI Certification Programs. ANSI, 1430 Broadway, New York, N.Y.

American Society for Testing and Materials (ASTM). 1977. Standard Practice for Generic Criteria for Use in the Evaluation of Testing and/or Inspection Agencies, ASTM Designation E548 A.S.T.M., 1916 Race St., Philadelphia, Pa.

Bicking, C. A., and W. E. Deming. 1971. A Reappraisal of the Contribution of Statistical Methods to Quality Assurance Proceedings. International Statistical Institute, Washington, D.C.

Center for Disease Control. 1975. Guide to Proficiency Testing. Atlanta, Ga.

FDA. 1976a. Compliance Program Manual. Transmittal No. 76-209, Food and Drug Administration.

FDA. 1976b. Proposed Regulations for Good Laboratory Practice. Food and Drug Administration. Fed. Regist. 41(225): 51206-51230.

Huibregste, K. R., and V. H. Moses. 1976. Handbook for Sampling and Sample Preservation of Water and Waste Water. EPA 600/4-76-049.

National Bureau of Standards. 1973. Clinical Laboratory Performance Analysis Using Proficiency Test Statistics. NBSIR 73-197 (Authors: P. W. Finkel and J. W. Rowen).

National Cancer Institute. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. NCI-CG-TR-1. Bethesda, Md.

National Research Council. 1972. Guide for the Care and Use of Laboratory Animals. DHEW No. (NIH) 74-023. U.S. Government Printing Office, Washington, D.C.

Office of the Secretary of Commerce. 1976. Procedures for a National Voluntary Laboratory Accreditation Program. Fed. Regist. 41(38): 8163-8168.

OSHA. 1974. Proposed Rules for Accreditation of Testing Laboratories. Occupational Safety and Hazard Administration. Fed. Regist. 39(191).

Slack, K. V., R. C. Averett, P. E. Grewson, and R. G. Lipscomb. 1973. Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples. Book 5, U.S. Department of the Interior, Washington, D.C.

U.S. EPA. 1975. Handbook for Evaluating Water Bacteriological Laboratories. Second Edition. U.S. Environmental Protection Agency, EPA-670/9-75-006, Cincinnati, Ohio.

U.S. EPA. 1976. Handbook for Sampling and Sample Preservation of Water and Wastewater. U.S. Environmental Protection Agency, EPA-600/4-76-049, Cincinnati, Ohio.

3.2 FIELD RESEARCH

3.2.1 Field Sampling

A general requirement for satisfactory sampling is valid and representative samples (McFarren, 1974).

A formal sampling plan includes:

- the selection of sampling site (Section 3.2.1.1)
- the frequency of sampling (Section 3.2.1.2)
- the calibration and maintenance of sampling equipment (Section 3.2.1.3)
- sample preservation (Section 3.2.1.4)
- the selection of sampling methods (Section 3.2.3)

3.2.1.1 Selection of Sampling Site--

The selection of the sampling site is the beginning, inevitable task of any field biologist. There is limited, scattered information on selection of sampling sites in field biology literature. The following criteria should be taken into consideration when selecting the sampling site:

- familiarity with historical data including biological, chemical, and physical nature of the site
- good definition of the study objective
- degree of accessibility
- whether or not the stands (or stations) appear to be representative
- availability of satisfactory adjacent stands, since it is convenient to establish more that one station at each field locality (Davis and Gray, 1966)
- organism-specific: For fish, sample in the obscure and unlikely areas as well as at obvious locations; sample all depths, not just surface and bottom. For other organisms, sample to suit the special requirements
- habitat-specific: In rivers, one sample upstream and another downstream from the pollution source. In lakes, reservoirs, and other standing-water bodies where the zones of pollution may be arranged concentrically, locate stations in an area adjacent to the waste outfall and in an unaffected area
- for aquatic vegetation: Three criteria are applied in the decision to include or reject a particular side (Auclair et. al., 1976). They are:

  o Following an initial survey prescribe samples in proportion to the area covered in each existing emergent vegetation type
  o Sample at different water depths
  o At Least one emergent species has to be present

- In benthic studies, station positions must be stratified to reflect both natural abiotic environmental gradients and pollutional gradients.

"Criteria for locating stations must receive more attention. Preliminary cruises should be designed simply to determine the position of future stations. Justifications for sampling grids should be included in all research reports" (Swartz, 1976).

Weber (1973) gave some suggestions on selecting sampling sites for plankton studies with regard to pollution. First of all, it was suggested that sampling be widespread to define the quantity and nature of all plankton in the body of water in long-term studies. In short-term studies, sampling sites might be more restricted because of limitations in time and manpower. Secondly, it was recommended to locate the sites upstream and downstream from a suspected pollution source in a small stream or river and to locate sampling sites in lakes, reservoirs, estuaries, and the oceans in grid networks or along longitudinal transects. Thirdly, if pollutants are discharged from various sources, locate sampling sites in such a manner as to separate their effects, i.e., antagonism, synergism or additivity. Finally, on the basis of historical data, choose sampling sites including areas from which plankton have been collected in the past.

For studying pesticide residues in the water environment, Lauer (1974) emphasized that the location of the sampling station must make it possible to obtain samples representative of the water body being sampled. The greater the variability of the water mass, the more sampling stations must be selected.

If the objective of a study is qualitative in nature (to describe the flora and fauna of an area with a high degree of accuracy), a relatively large number of samples must be collected from a large number of habitat types (Slack et al., 1973).

3.2.1.2 Frequency of Sampling--

Frequency of sampling is of critical importance. In a sampling program, it evidently influences the validity of data, particularly the precision and accuracy of data. In general, the more frequent the sampling, the more precise and accurate the data.

There are many elements that determine the frequency of sampling. Among these elements some important ones are:

- the objective of study
- the organisms being studied
- the availability of manpower
- the availability of historical information
- the limitation of time
- the limitation of money
- the adequacy of sampling equipment
- environmental factors

Prior to the beginning of sampling, study objectives must be defined clearly and carefully. For example, the frequency of sampling may vary from hourly, for a detailed study of diel variability, to quarterly (every third month), for a general estimation of seasonal variations, depending on the objectives (Rand et al., 1975). If the objective of the study is quantitative in nature, the increase in the frequency of sampling may increase the precision of the data., e.g., of the estimation of fish population in a body of water. Frequent samplings are also necessary in a pollutional study if the characteristics of effluents change or if spills occur. This will help biologists more precisely to locate the effluents or spills in a given study area.

Available manpower, time and money always determine the scope of study and the frequency of sampling, too. The sampling frequency must be adjusted to limitations in personnel, time and money.

If sampling can be automated, more frequent samplings can be made than are otherwise possible. For example, automatic monitors can sample air, water or other media continually, e.g., hourly through day, month, and year.

Biological factors such as organisms being studied determine the frequency of sampling. That is, the frequency of sampling for plankton may differ from that for fish or other organisms because each studied organism has its unique biology, e.g., habitat types, and natural variability.

The frequency of sampling is sometimes determined by the available historical information attainable by searching literature (or work) by previous investigators.

Environmental factors may also influence the determination of sampling frequency. For instance, sudden meteorological changes such as a hurricane storm may force biologists to sample more frequently in its aftermath.

3.2.1.3 Calibration and Maintenance of Sampling Equipment--

Table 3.2.1 summarizes the equipment used currently in biological field sampling. It is generally agreed that no type of sampling equipment is applicable to all biological communities. Instead, there is sampling equipment available for each biological community, such as the special nets, pumps and water bottles applicable to a plankton community.

Traditionally, little importance is attached to the calibration of field sampling equipment. This is probably due to two things:

- most field sampling equipment is designed for qualitative studies
- for quantitative studies, sampling frequency and site selection affect the precision of data much more than calibration errors

TABLE 3.2.1 A LIST OF BIOLOGICAL SAMPLING EQUIPMENT (U.S.EPA, REGION II, 1975; U.S.DI, 1972)

Organisms	Field Sampling Equipment
Mammals	Mouse traps (mouse, rat, etc.)
	Conibear traps (bear, etc.)
	Snares (deer, etc.)
	Box traps (chipmunk, muskrat, etc.)
	Beaver traps (beaver)
	Herd traps (deer, etc .)
	Nets (monkeys, etc.)
	Guns (rabbits, deer, etc.)
Birds	Box or enclosure traps (gregarious seed-eaters)
	Net or rocket trap (wild turkey, etc.)
	Drive and drift traps (water fowl)
	Mist nets (commercial birds)
	Nest traps (water fowl)
Reptiles	Drift traps (snakes)
Plants	Transportation
	Survey gear
	Base maps
	Specimen containers
Fish	Electric shocker
	Gill nets
	Trammel nets
	Seines
	Trawls
	Others (hook and line, chemicals, etc.)
Macroinvertebrates	Grab samplers (Ponar, Peterson, Ekman, Tall Exman, Orange Peel, Shipek, Smith-McIntyre, etc.)
	Surber sampler
	Corers
Zooplankton	Nets
	Clark-Bumpus
	Pumps
	Integrated (tubular) samplers
	Kemmerer or Van Dorn water bottles
	Juday trap
Periphyton	Artificial substratum
	Natural substratum

(continued)

TABLE 3.2.1 (Continued)

Organisms	Field Sampling Equipment
Phytoplankton	Nets Clark-Bumpus Pumps Integrated (tubular) sampler Kemmerer or Van Dorn water bottles Others
Macroalgae (e.g. chara)	Same as aquatic plants
Macrophytes (Aquatic vascular plants and aquatic plants)	Transportation gear of survey Base maps Specimen containers
Microorganisms	Water sampling bottle, e.g., Kemmerer type

The following information on equipment used ought to be included on the field data sheet:

- date of use
- user's name
- operating conditions
- special remarks on maintenance and repair

For sampling equipment, maintenance and repair are more important than anything else. Regular maintenance work consists of:

- good, thorough cleaning after use
- drying before storage
- proper storage

For example, nets for plankton or fish collection need attention. In particular, sampling equipment employed in brackish or marine water requires a freshwater rinse to prevent rusting or rotting. The repair of equipment should be scheduled and done on time and by the right personnel. Replacement must be considered if repaired equipment does not do an adequate sampling job.

The maintenance of mobile laboratory facilities should be also considered as an important task.

3.2.1.4 Sample Preservation--

Upon obtaining a valid and representative sample in the field, sample preservation is an important consideration. Biological sample preservation normally emphasizes:

- sample holding container
- type of preservative used
- sampling labelling information
- holding time between sampling and analysis

The containers for biological sample material can be divided into two principal categories: glass and plastic. There are many types in each category. All containers have their disadvantages. In general, the major disadvantage of glass is that it is breakable and heavy-weight. This can be a strict handicap in field use. Polyethylene, on the other hand, is durable, light-weight and easy to handle. So plastic containers are more widely accepted. Nevertheless, both kinds of containers require the use of proper chemicals to preserve field biological materials.

The chemical preservatives most often used for general field preservation include formaldehyde, ethyl alcohol, borax, and arsenic trioxide. The use of these preservatives for organisms varies from microscopic protozoa to large mammals. Table 3.2.2 presents recommended techniques for using these preservatives with a number of biological materials. Each recommended technique is briefly described.

As shown in Table 3.2.2, in addition to chemical preservation, physical preservation of biological material is also recommended. Two means are employed in physical preservation: refrigeration and freezing. Refrigeration (approximately 1-2°C) is an excellent way to preserve most biological materials for a short period of time. For longer periods of preservation deep freezing (approximately -20°C) is considered as an excellent method to preserve many specimens. Either way, it must be kept in mind that the specimen should be placed in a watertight container, e.g. a plastic bag, and packed in a second container with either dry or natural ice surrounding the inner-most container. The sample (or specimen) must be shipped immediately to a central laboratory for analysis.

TABLE 3.2.2 TECHNIQUES RECOMMENDED FOR PRESERVATION OF BIOLOGICAL MATERIAL (MOSBY AND COWAN, 1971)

Biological Material	Recommended techniques, listed in order of preference
Mammals	
whole, small	(1) Ethyl alcohol (70%); (2) 5% Formalin
whole, large	Formalin (7-10%); also injection of preservative into internal organs by hypodermic-perfusion via circulatory route

(continued)

TABLE 3.2.2 (Continued)

Biological Material	Recommended techniques, listed in order of preference
Skins, pelts	(1) Clean thoroughly and air dry; (2) clean and salt thoroughly (NaCl); (3) use alum on pelts which appear to be "slipping"
Skins, study*	(1) Borax (not to be used on skins having red pelage); (2) arsenic trioxide-borax mixture in equal proportions; (3) arsenical soap
Food material stomachs	Small stomachs-5% Formalin; large stomachs-5 to 10% Formalin (wrap stomachs in cheesecloth)
Droppings	Dry quickly, fumigate with carbon disulfide
Reproductive tracts	(1) AFA (preferably) or Bouin's fluid; (2) 10% Formalin
Birds	
whole	(1) 70% alcohol; (2) 5% Formalin, both with internal injection
Skins, pelts) Skins, study)	(1) Borax; (2) arsenic-borax mixture
Stomachs	5% Formalin
Droppings and pellets	Dry quickly and fumigate with carbon disulfide
Reptiles & Amphibians	
whole	(1) 35-40% isopropyl alcohol or 70% ethyl alcohol; (2) Formalin-specimens should be slit or injected
Snake skins	Rolled flat, placed in 70% alcohol
Salamanders	Kill with chloretone or 20% alcohol; harden with 5% Formalin and store in 70% alcohol
Amphibian skins (to preserve color)	Kill with ether; skin and place skin in water; float onto cardboard; dry quickly
Fish	(1) 70% alcohol; (2) 10% Formalin
Insects	
Hard bodies	Kill with KCN bottle; store dry
Soft bodies	Kill and store in 5% Formalin or 10% alcohol

(continued)

TABLE 3.2.2 (Continued)

Biological Material	Recommended techniques, listed in order of preference
Miscellaneous	
Skeletons-field	(1) Clean thoroughly and dry quickly; treat with arsenical soap† for shipment; (2) place in alcohol (Formalin, unless neutralized, dissolves calcium of bones)
Skeletons-in laboratory ‡	(1) Boil gently in 3% hydrogen peroxide to remove meat and to bleach bones, degrease in carbon tetrachloride; (2) clean by use of dermestid beetles
Fumigants-for all specimens in pelt, study or standing mount form	Carbon disulfide as gas insecticide to kill insects; paradichlorobenzene as insect deterrent and DDT as insect contact killer
Pathological Material	
General	(1) Refrigerate (30°-40°F); (2) deep freeze and transport to laboratory as quickly as possible
Hematological	(1) Make several blood or tissue smears; (2) blood serum; (3) cell counts: either sodium oxalate 2-4 mg/ml or sodium citrate 2-4 mg/ml; refrigerate; (4) whole blood or serum dried on paper discs
Bacteriological	(1) Refrigerate entire specimens; (2) take blood, pus or fluids in sterile containers; refrigerate; (3) saturate cotton swabs with blood, pus, or tissue juices; transport in special medium; (4) make smears from blood, serous fluids, tissue juices
Virological	
Rabies	If possible confine the animal and wait until death occurs. Refrigerate and rush the head (if possible the entire carcass) to Public Health Laboratory
Other Viruses	(1) refrigerate; (2) freeze; (3) put 1 cm cubes of tissue in glycerol
Parasitological	
Ectoparasites	Remove by hand or with aid of ether, chloroform, or sorptive silica powder (Dri Die). (1) ship live in non-airtight container with moist cotton, refrigerate if possible; (2) kill with ether, chloroform or HCN and ship dry between layers of cotton; (3) freeze and ship frozen

(continued)

TABLE 3.2.2 (Continued)

Biological Material	Recommended techniques, listed in order of preference
Helminths	(1) relax in cold water. Fix nematodes in hot 70% alcohol. Fix cestodes, trematodes in warm AFA; (2) 70% alcohol-95 parts, glycerol-5 parts
Protozoa	(1) refrigerate tissues, feces, citrated blood (2) make smears of blood, feces, tissue impressions; (3) fix tissues in 10% Formalin
Histological	Fix small pieces of tissue in 10% Formalin (10 to 20 x volume of tissue). Do not freeze
Toxicological	Refrigerate or freeze blood, liver, kidneys, brain, stomach with contents, small intestine
Plants	
Terrestrial	Place between folded paper, dry quickly between corrugated cardboards and with slight pressure in plant press
Aquatics, or other plants with a mass of tissue	(1) alcohol-acetic acid-Formalin solution; (2) 2 to 4% Formalin

* Injection with embalming fluid (equal parts of Formalin, glycerine, and phenol plus 85 parts water) will keep birds and mammals fresh enough to skin for study mounts for about a week without refrigeration.

† Poisons should not be used on skeletons which are to be cleaned by dermestids.

‡ Clean large skulls and skeletons by boiling in 4 oz. sodium sulfate and 8 oz. ammonia to 6 gallons of water.

Samples are useless unless adequately labelled. The samples or sample containers must have attached the following information, written with a waterproof marker on durable paper:

- date
- name of study area
- site of sampling station
- type of sample (qualitative or quantitative)
- volume of water represented or weight where applicable
- number of subsamples of sample
- type of analyses desired for sample
- name of collector
- method of sample collection

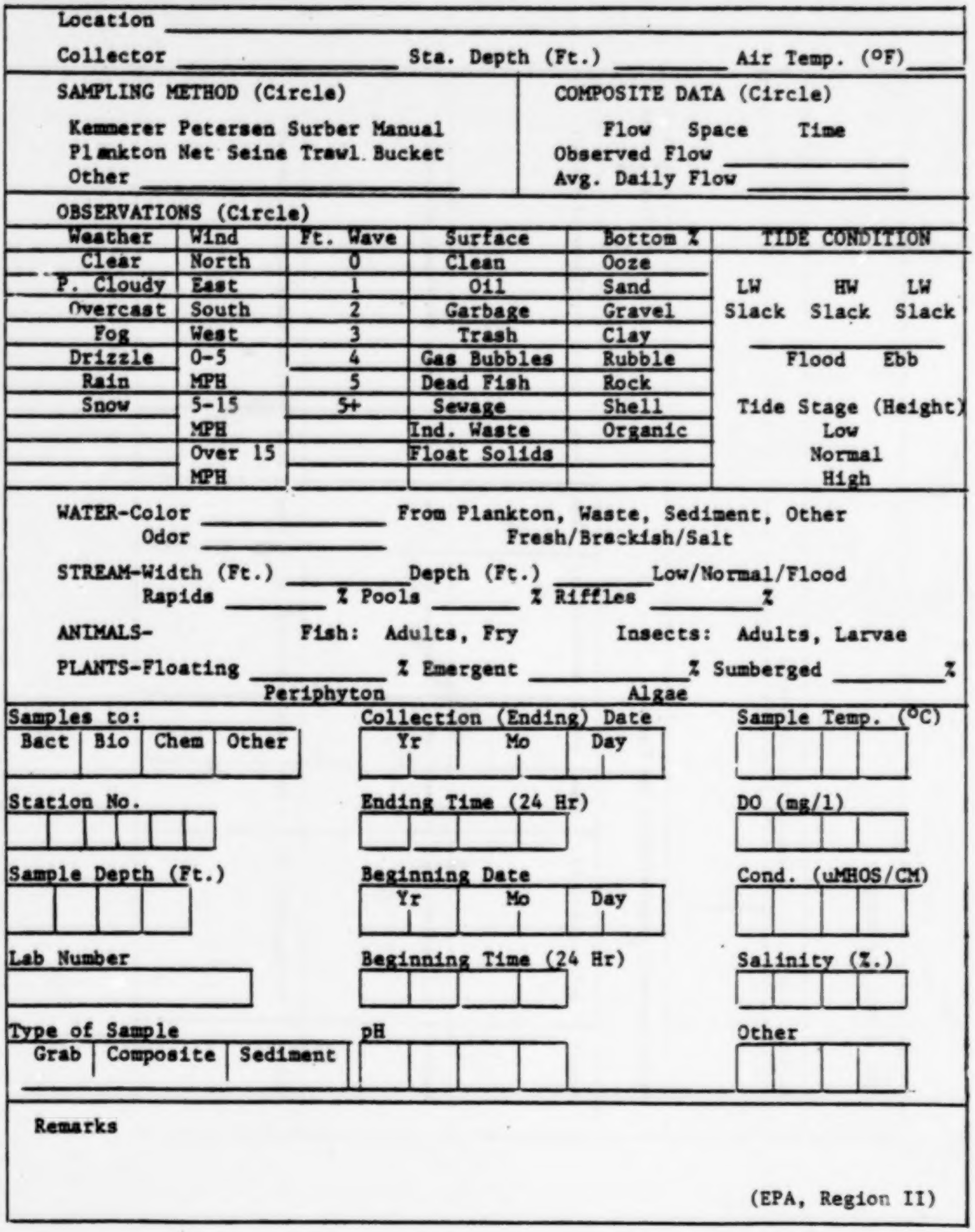
It is always a good practice to duplicate full data on a second label and to pack it with the sample container so that at least one set of sample information is preserved. This labelled sample should go to the project manager or laboratory supervisor with a completed field data sheet (Figure 3.2.1) and a completed chain of custody form (Figure 3.2.2).

Holding time has been defined in the following ways:

- the entire period of time from the point of the initial sample collection to the beginning of the analysis
- the period of time between the point of receipt of the sample at the laboratory and analysis
- the period of time between the point of the formation of composite sample and analysis

Neither of the latter two show the real length of time a sample has been moved away from its environment. Therefore, the results of analysis may not be valid due to the inaccurate reflection of possible changes. This may be critical when analyzing for the population of microbiologic bacteria which change fast in water, but it may not be important for fish scale samples that are commonly preserved for age and growth study. Consequently, the holding times between the beginning of sampling in the field and analysis in the laboratory must be specified.

Under no circumstances should the laboratory supervisor or project manager delay the analyses on any field biological samples. When a sample enters the laboratory, the material with the shortest holding time should be analyzed first. In the meantime, a composite sample must be formed if needed and further preservation in the laboratory must be accomplished if required. Then the relatively stable samples can be analyzed. Thus, the problem of delayed analyses is reduced to a minimum.



Location ______________________________

Collector ______________ **Sta. Depth (Ft.)** ________ **Air Temp. (°F)** ____

SAMPLING METHOD (Circle)

Kemmerer Petersen Surber Manual
Plankton Net Seine Trawl Bucket
Other ____________________

COMPOSITE DATA (Circle)

Flow Space Time
Observed Flow ____________
Avg. Daily Flow ____________

OBSERVATIONS (Circle)

Weather	Wind	Ft. Wave	Surface	Bottom %	TIDE CONDITION
Clear	North	0	Clean	Ooze	
P. Cloudy	East	1	Oil	Sand	LW Slack HW Slack LW Slack
Overcast	South	2	Garbage	Gravel	
Fog	West	3	Trash	Clay	
Drizzle	0-5	4	Gas Bubbles	Rubble	Flood Ebb
Rain	MPH	5	Dead Fish	Rock	
Snow	5-15	5+	Sewage	Shell	Tide Stage (Height)
	MPH		Ind. Waste	Organic	Low
	Over 15		Float Solids		Normal
	MPH				High

WATER-Color ____________ From Plankton, Waste, Sediment, Other
Odor ____________ Fresh/Brackish/Salt

STREAM-Width (Ft.) ________ Depth (Ft.) ________ Low/Normal/Flood
Rapids ________ % Pools ________ % Riffles ________ %

ANIMALS- Fish: Adults, Fry Insects: Adults, Larvae

PLANTS-Floating ________ % Emergent ________ % Sumberged ________ %
Periphyton Algae

Samples to: Bact | Bio | Chem | Other

Collection (Ending) Date: Yr | Mo | Day

Sample Temp. (°C)

Station No.

Ending Time (24 Hr)

DO (mg/1)

Sample Depth (Ft.)

Beginning Date: Yr | Mo | Day

Cond. (uMHOS/CM)

Lab Number

Beginning Time (24 Hr)

Salinity (%.)

Type of Sample: Grab | Composite | Sediment

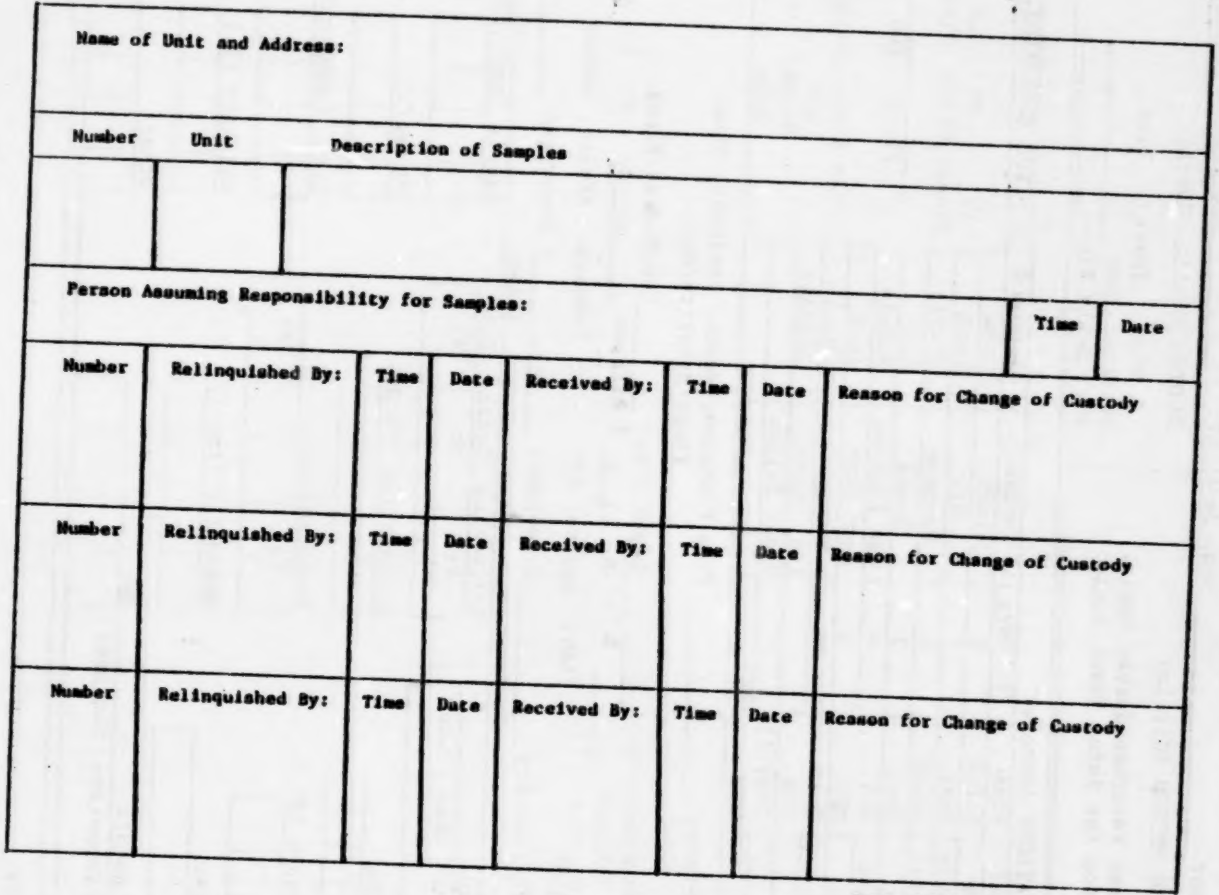
pH

Other

Remarks

(EPA, Region II)

Figure 3.2.1 Field data sheet (U.S. EPA, Region II)



Name of Unit and Address:							
Number	Unit	Description of Samples					
Person Assuming Responsibility for Samples:						Time	Date
Number	Relinquished By:	Time	Date	Received By:	Time	Date	Reason for Change of Custody
Number	Relinquished By:	Time	Date	Received By:	Time	Date	Reason for Change of Custody
Number	Relinquished By:	Time	Date	Received By:	Time	Date	Reason for Change of Custody

(EPA, REGION II)

Figure 3.2.2 Chain of custody form (U.S. EPA, Region II).

3.2.2 Field Analysis

Biologists and analytical chemists have become more and more interested in having analyses done in the field because the holding and preservation of samples have been shown to affect the quality of results, i.e. the accuracy of data. For example, the addition of the common preservative, $HgCl_2$, that is applicable to the measurement of nutrients in the sample, interferes with the measurement of BOD (Biological Oxygen Demand). The bacterial inhibition by such chemicals reduces the BOD reading. For biological material, the preservation of most samples changes the original natural colors of organisms. This change sometimes makes it more time-consuming and more tedious to identify organisms. The prolonged holding of the preserved samples (especially in ethanol) often causes an underestimate of pesticide residues. Lauer (1974) recommended the routine check of pesticides in the preservative with the result to be added to the total obtained from the organisms before final computation of concentrations. Because of considerations like this, the Task Group on Biologic Quality and Organics of the Federal Intraagency Work Group on Designation of Standards for Water Data Acquisition suggested that some analyses that are usually done in the laboratory be practiced in the field (U.S. Department of Interior, 1972).

3.2.2.1 EPA Field Methods--

The U.S. Environmental Protection Agency (1973) has published a manual, entitled "Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents" (C.I. Weber, editor). It contains field and laboratory methodology for sampling, identifying and quantifying plankton, periphyton, macrophyton, macroinvertebrates, fish and bioassay, and has a chapter on "Biometrics". The manual is periodically reviewed, and revised existing methods and new methods are added as the need arises. The second edition of the EPA Methods Manual will be significantly expanded to include the following additional materials:

- Non-parametric statistical analysis
- Adenosine triphosphate analysis
- Nitrogen fixation (acetylene reduction) methods
- Liquid scintillation techniques for primary productivity
- Periphyton primary productivity methods
- Sediment oxygen demand
- Scuba techniques
- Histopathology and histochemistry
- Acetycholinesterase analysis
- Effluent bioassay
- Field and laboratory biological quality assurance guidelines

3.2.2.2 Instrument Calibration--

Table 3.2.3 lists instruments and equipment commonly used in the biological field analysis.

TABLE 3.2.3 INSTRUMENTS AND EQUIPMENT FOR LABORATORY AND FIELD ANALYSIS IN BIOLOGICAL RESEARCH

ATP photometers
Audial instruments
  (recorders, etc.)
Balances
Current meters
DO probes
Drying ovens
Fluorometers
Freezers
Gas chromatographs
Incubators
Light meters
Microscopes:
  Compound
  Electron
pH meters
Refrigerators
Salinometers
Spectrophotometers
Thermometers
Visual instruments
  (binoculars, etc.)
Volumetric glassware

Calibration procedures for spectrophotometers are described in "Spectrophotometer Calibration and Performance", ASTM E225-67. Rand et al. (1975) detail procedures for the calibration of microscopes, fluorometers, analytical balances and other instruments. According to Rand et al. (1975), balances shall provide a sensitivity of at least 0.1 g at a load of 150 g, with appropriate weights. An analytical balance having a sensitivity of 1 mg under a load of 10 g shall be used for weighing small quantities (less than 2 g) of materials. Single-pan rapid-weight balances are most convenient. See Chapter 3, Instrumental Quality Control, of the U.S. EPA's Handbook for Analytical Quality Control in Water and Wastewater Laboratories (U.S. EPA, 1972).

A good calibration system for any instrument for field and laboratory analysis should be based on the following requirements:

- Develop a calibration plan and follow it
- Use calibration standards. For example, solutions containing chlorophyll a, b and c and the degradation product pheophytin a are available for spectrophotometric analysis by writing to: U.S. Environmental Protection Agency, EMSL - Laboratory Evaluation and Quality Assurance Branch, 26 West St. Clair Avenue, Cincinnati, Ohio 45268. A quality control sample is also available from the above address for fluorometric analysis for chlorophyll
- Adequate environmental conditions should be provided during calibration
- A calibration interval for recalibration should be assigned to all instruments and equipment listed in Table 3.2.3 and calibration standards should be specified
- A record of calibration should be maintained for each instrument or piece of equipment. This record consists of:

  - Date
  - True value of standards and calibration value
  - Factor, if any required to correct reading from meter
  - Amount of drift
  - Initials of person performing calibration

- Written calibration procedures should be provided for all listed instruments and equipment. These are usually collected in a quality

control manual
- The calibration record should include the traceability of the standard used in the calibration
- A calibration checklist should be provided and instruments and equipment should be checked and adjusted periodically by a laboratory service man or consultant if service is not available locally, following manufacturer's instructions as closely as possible

3.2.2.3 Field Sampling with Laboratory Analysis--

Assuming that all samples are collected properly in the field and handled adequately, and field analysis is not dictated by preservation problems, laboratory analysis can then be initiated by the project manager.

The analysis of samples is basically in two groups: qualitative and quantitative analysis. Table 3.2.4 lists the major analyses for field-collected samples of common organisms. Qualitative analysis is primarily for organisms and species identification. Quantitative analysis includes other functional tests such as:

- Number
- Productivity
- Growth
- Bioassays
- Chemical analyses (tissue analyses)

Recently, the taste test (flesh tainting) of commercial macroinvertebrates and fish has come into the territory of laboratory analysis.

In a broad sense, bioassay can be divided into field bioassay and laboratory bioassay. Most biologists are familiar with laboratory bioassay which in general comprises aquatic and mammalian bioassay. Aquatic and mammalian laboratory bioassays are discussed in Section 3.3 and 3.5, respectively. Field bioassays will come under further discussion later in this section.

TABLE 3.2.4 MAJOR ANALYSES OF COMMON ORGANISMS IN FIELD SAMPLING WITH LABORATORY ANALYSES

Organisms	Major Analysis
Viruses	Identification
Bacteria	Identification Colony count
Phytoplankton	Identification and counts Diatom species proportional count Ash-free weight Chlorophyll analyses

(continued)

TABLE 3.2.4 (Continued)

Organisms	Major Analysis
	ATP determinations Primary productivity Bioassay
Zooplankton	Identification and counts Dry weight Ash-free weight Bioassay
Periphyton	Identification and counts Diatom species proportional counts Ash-free weight Chlorophyll analyses ATP determinations Primary productivity Bioassay
Macrophyton	Identification Dry weight Ash-free weight Chlorophyll analyses Bioassay
Macroinvertebrates	Identification and counts Dry weight Ash-free weight Age and growth Bioassay
Fish and other vertebrates	Identification and counts Age determinations Growth measurement (in length and/or weight) Bioassay
Plants	Identification Dry weight Ash-free weight Bioassay

Chemical analysis is usually done to determine the amount of three groups of major environmental contaminants, pesticides, metals, and radioisotopes, in each trophic level of organisms. Tissues are often employed for histopathological analysis and histochemical (or enzyme) analysis.

3.2.3 Sampling Method

This section covers sample collection, sample preparation, preservation and storage, and sample analysis for the following test subjects (for viruses and bacteria, see Section 3.4.1):

- Plankton
- Periphyton
- Macrophyton
- Macroinvertebrates
- Fish
- Birds
- Mammals
- Plants

3.2.3.1 Plankton--

In "Biological Field and Laboratory Methods" (U.S. EPA, 1973), plankton is defined as organisms suspended in a body of water which, because of their physical characteristics or size, are incapable of sustained motility in directions counter to the water currents. In fresh water they are generally microscopic; in sea water, they are more frequently larger. All of them drift with currents.

Plankton consists of both plants and animals. The planktonic plants are referred to as "phytoplankton" and animals are "zooplankton". Reports have shown that complex and intimate relationships exist among the various components of plankton. Phytoplankton such as algae occur as unicellular, colonial, or filamentous forms, and usually constitute the greatest portion of the biomass of plankton. These chlorophyll-bearing plants carry on photosynthesis and serve as primary producers. The zooplankton in fresh water comprise primarily protozoans, rotifers, cladocerans, and copepods; in marine waters, a much greater variety of organisms is encountered. Zooplankton and other herbivores graze upon the phytoplankton and, in turn, are preyed upon by other organisms, thus passing the stored energy along to larger and usually more complex organisms. In this manner nutrients become available to large consumers such as macroinvertebrates and fish.

For the following reasons, plankton have been used extensively by pollution engineers and biologists as indicator organisms for environmental-assessment studies (Rand et al., 1975):

- Because of their short life cycles, plankton responded sensitively to environmental changes, and hence the species composition and standing crop indicate the quality of the water mass in which they reside

- Because of their small size and great numbers, they not only strongly influence certain non-biological aspects of water quality (e.g., pH, color, taste, and odor), but in a practical sense, they are a part of water quality. However, because of their transient nature, plankton communities may be of limited value in assessing water quality

The decision on selecting the sites and stations for plankton samples should be made according to the following:

- Formulate a study design which includes study objectives, the limitations of manpower, time and money
- Select the same sampling sites selected by previous investigators if consistent with study aims, for a better understanding of current results
- Select the sampling stations as near as possible to those selected for chemical and bacteriological sampling to insure maximum correlation of findings
- Select a sufficient number of stations in as many sites as necessary to define adequately the kinds and quantities of plankton in the waters studied
- Understand the physical nature of water (such as currents, depths, and volume of flow) that influences greatly the selection of the sampling stations

Keeping of field notes and inserting of sampling labels must be taken into consideration in plankton collection. Both labels and marker should be waterproof. Record the following information on all labels:

- Sample identification number
- Location, including name of water body, distance and direction to nearest city, county and state, latitude and longitude, or other description
- Date and time
- Name of collector
- Type of sample, including equipment used, sample volume, tow length if net is used, vertical or horizontal tow
- Preservatives used and concentrations
- Special preparation of samples desired
- Types of analyses to be performed, as a reminder and a cross-check

Keep a field notebook containing all information written on the label, plus pertinent additional notes. These notes should include, but not be limited to:

- Weather conditions: wind direction and intensity, and cloud cover
- Physical nature of water: smooth water surface or rippled, water color and turbidity, and depth at station

- A list of all types of samples taken at station
- Information on direction, distance, and description of effluents in the vicinity
- Other general descriptive information

Sample size depends on the type and number of determinations to be made; the number of replicates depends on the statistical design of the study and the statistical analyses selected to assist in data interpretation (Rand et al., 1975).

TABLE 3.2.5 PRESERVATION OF PHYTOPLANKTON

Preservatives	Preparation	Usage
Formalin plus sodium tetraborate (neutralized Formalin)	Neutralize Formalin with tetraborate to pH=7.0-7.3. Five milliliters of the neutralized formalin are added for each 100 ml of sample	Preserve the samples for more than 1 year, but this preservative will cause many flagellated phytoplankton to lose flagella
Neutralized Formalin plus cupric sulfate	Add saturated cupric sulfate solution to the preserved samples. One milliliter of the saturated solution per liter of sample is adequate	Maintains the green color of phytoplankton samples and aids in distinguishing photoplankton from detritus
Neutralized Formalin plus detergent solution	One part of surgical detergent to five parts of water makes a stock solution. Add 5 ml of stock per liter of sample	Prevents clumping of settled organisms
Merthiolate	Dissolve 1.0 gram of merthiolate, 1.0 gram of aqueous saturated iodine-potassium iodide solution (prepared by dissolving 40 grams of iodine and 60 grams of potassium iodide in 1 liter of distilled water), and 1.5 gram of borax in 1 liter of distilled water. Add 37.3 ml of this stock solution to 1 liter of sample	Stain cell parts to simplify identification. But this preservative will cause blue-green algae to lose gas from their vacuole and so enhances settling

EXAMPLE: PHYTOPLANKTON

Sample Collection

- Sample equipment: Nets, pumps, tubular equipment and cylindrical type of samplers are generally used for phytoplankton sampling (see Table 3.2.1). However, the U.S. Environmental Protection Agency has recommended the use of the cylindrical type of sampler with stoppers (U.S. EPA, 1973). Net collection of phytoplankton is recommended for quantitative analysis. Pumping may harm delicate algae when tubing is flushed between stratified samplings.
  QUALITY CONTROL -- Use only nonmetallic samplers when metal analysis, algae assays, or primary productivity measurements are being performed.

- Sample volume: When phytoplankton densities are less than 500 units per milliliter collect a 6-liter sample. In richer waters, a sample of 1 to 2 liters is sufficient.
  QUALITY CONTROL -- For quantitative analysis, caution must be taken to be exact on sample volume.

- Sample preservation: See Table 3.2.5 for preservatives used, their preparation and usage. Each preservative has its advantages.
  QUALITY CONTROL -- When diatom slides are to be made, DO NOT use detergent solution which prevents clumping of settled organisms.
  QUALITY CONTROL -- If merthiolate is used as preservative, the preserved samples are not sterile, and SHOULD NOT be stored for more than 1 year. After that period of time, Formalin should be used.

- After collection and preservation, phytoplankton samples sometimes must be concentrated in the laboratory before analysis. Three common techniques used for concentrating are: sedimentation, centrifugation, and filtration. Sedimentation is preferred (U.S. EPA, 1973). Because of the different sedimentation rates of the various sizes and shapes of phytoplankton, caution must be exercised during sedimentation.

- From the sample concentrates, a subsample is always withdrawn for phytoplankton semipermanent wet mounts, phytoplankton membrane filter mounts, or diatom mounts. See Standard Methods, 14th edition (Rand et al., 1975) for the detailed preparation of mounting slides. The mounted slides will be ready for microscopic examination for species composition and count.

Sample Analysis

- Qualitative analysis--Phytoplankton identification: Identify the phytoplankton to species level whenever possible. When identifying phytoplankton, it is useful to examine fresh, unpreserved samples. An initial examination is needed because most phytoplankton samples contain a diverse gathering of organisms.
  QUALITY CONTROL -- Use a good quality compound binocular microscope with a mechanical stage. Require a substage condenser for high magnification.
  QUALITY CONTROL -- For exact magnification, the microscope must be adequately calibrated.

QUALITY CONTROL -- Utilize all available references for exact identification and consult the authority for questionable identification.

- Quantitive analysis-Phytoplankton count: Identify and count the phytoplankton directly. In samples with very low populations, concentrate organisms and then count. In those samples where algae concentrations are extreme, or where silt or detritus may interfere, carefully dilute a small portion of the sample 5 to 10 times with distilled water, and then count. The apparatus (five types) used in counting phytoplankton are listed below. For procedures of using each apparatus, see Standard Methods, 14th edition (Rand et al., 1975), or Biological Field and Laboratory Methods (U.S. EPA, 1973).
  QUALITY CONTROL -- Use an adequately calibrated microscope.
  QUALTIY CONTROL -- The analyst should carefully manipulate the dilution and concentration of the samples that may introduce error.

- (1) Sedwick-Rafter (S-R) cell is 50 mm long by 20 mm wide by 1 mm deep and the total volume is 1000 mm^3 or one ml.
  QUALITY CONTROL -- The diluted or concentrated samples must be well mixed before transfer into counting chamber.
  QUALITY CONTROL -- Be exact on the volume of the well-mixed sample to be transferred into the chamber, e.g., 1.0 milliliter.
  QUALITY CONTROL -- Examine the underside of the cover slip and add these organisms to the total count.
  QUALITY CONTROL -- Always randomly select the strips or fields for count.
  QUALITY CONTROL -- Be consistent on counting phytoplankton that lie only partially within the grid or that touch one of the edges.

- (2) Palmer-Maloney (P-M) Nannoplankton cell: The cell has a circular chamber 17.9 mm in diameter and 0.4 mm deep, with a volume of 0.1 ml.
  QUALITY CONTROL -- Use P-M cell only for nannoplankton count.

- (3) Bacterial Counting cells and Hemocytometers: The cell (Petroff-Hausser cell) is 1 mm x 1 mm x 1/50 mm which gives a volume of 1/50 mm^3. The depth in the hemocytometer is 1/10 mm (compared to 1/50 mm in a P-H cell), and thus the total stabilization volume is 1/10 mm^3.
  QUALITY CONTROL-- Do not attempt routine counts until experienced in use of the bacterial counter and the statistical validity of the results is satisfactory.
  QUALITY CONTROL -- Employ these cells for counting high-density populations (50,000 cells/ml) that may be found in sewage ponds or in laboratory cultures.

- (4) Membrane Filter: A special filtration apparatus using a vacuum of 0.5 atmospheres and 1-inch, 0.45 μ membrane filters.
  QUALITY CONTROL -- Be exact on the amount of water to be filtered.
  QUALITY CONTROL -- The filtered samples from estuarine and sea waters must be rinsed with distilled water to remove salts.
  QUALITY CONTROL -- Record the occurrence of each species in 30 random fields.

- (5) Inverted microscope with cylindrical counting chambers: Precision-made, all-glass counting chambers in a wide variety of dimensions are available. The chambers can also be easily and inexpensively made in the laboratory.
  QUALITY CONTROL -- Allow complete sedimentation before making a count. On the average, allow 4 hours per mm of height.
  QUALITY CONTROL -- Make random counts. For field counts, as a general rule, count a minimum of 100 of the most abundant species. At higher magnification, count more fields than under lower power.

- Diatom Analysis: Identification and Count. Prepare diatom slides as directed in Standard Methods, 14th edition (Rand et al., 1975) or Biological Field and Laboratory Methods (U.S. EPA, 1973). Identify and count the diatoms at high magnification under oil. Randomly examine lateral strips the width of the Whipple grid, and identify and count all diatoms until 250 cells are counted.
  QUALITY CONTROL -- The slides must be labelled with all relevant information.
  QUALITY CONTROL -- Use "A Guide to the Common Diatoms at Water Pollution Surveillance System Stations", as a basic reference (Weber, 1971). Utilize all other available references and experts for identifying purposes.
  QUALITY CONTROL -- Adopt a consistent system on counting. Count all diatoms within the borders of the grid. Ignore small cell fragments.

- There are two other counting methods for quantifying phytoplankton: Lackey Drop Microtransect Counting Method; and Particle Counters (Rand et al., 1975; Lackey, 1938; Maddux and Kanwisher, 1965). The former method is a simple method of obtaining counts of considerable accuracy with samples containing a dense plankton population. It is similar to the S-R strip count. The particle counters are used effectively for counting pure culture but are not suited for enumerating natural plankton populations in surface water grab samples because they do not discriminate between the plankton and other particles such as silt or organic detritus.

- Biomass determination: Chlorophyll can be measured in vivo fluorometrically or in acetone extracts (in vitro) by fluorometry or spectrophotometry. The measurements can be categorized into four types: (1) spectrophotometric determination of chlorophyll a, b, and c (Trichromatic Method), (2) fluorometric method for chlorophyll a, (3) spectrophotometric determination of pheophytin a (a common degradation product of chlorophyll a), and (4) fluorometric determination of pheophytin a.
  QUALITY CONTROL -- Keep the stored samples in the dark to avoid photochemical breakdown of the chlorophyll.
  QUALITY CONTROL -- Mix the phytoplankton sample thoroughly to ensure a homogenous suspension of algal cells (in vivo measurement).
  QUALITY CONTROL -- Calibrate the spectrophotometer or fluorometer with calibration standards. See Section 3.2.2.2.
  QUALITY CONTROL -- Stopper the cuvettes to minimize evaporation of acetone during the time the spectrophotometric or spectrofluorometric readings are being made (in vitro measurement).

- See Standard Methods, 14th Edition (Rand et al., 1975) for details concerning equipment and reagents used, procedures, and calculations.

- Phytoplankton productivity measurements indicate the rate of conversion from inorganic carbon to an organic form by phytoplankton during photosynthesis. These measurements are useful in determining the effects of pollutants and nutrients on the aquatic community (U.S. EPA, 1973). Two widely used methods of measuring phytoplankton productivity in situ are: the oxygen method of Gaarder and Gran, and the carbon-14 method of Steeman-Nielsen.
  QUALITY CONTROL -- DO NOT use phosphorus-containing detergents to clean BOD bottles. Acid-clean them, JUST BEFORE use, rinse with the water being tested.
  QUALITY CONTROL -- Double precaution must be taken to insure light exclusion of the dark bottles used.
  QUALITY CONTROL -- Build supporting line or rack that DOES NOT shade the suspended bottles.

- (1) Productivity, Oxygen Method: See Rand et al. (1975), pp. 1037-1039 and 440-454.
  QUALITY CONTROL -- Water used to fill duplicate clear, darkened, and initial-analysis bottles SHOULD come from the same grab sample.
  QUALITY CONTROL -- Incubate the BOD bottles for at least 2 hours, but NEVER longer than it takes for oxygen-gas bubbles to form in the clear bottles.

- (2) Productivity, Carbon-14 Method: General directions for this method are found in Rand et al. (1975), pp. 1039-1041, pp. 278-282, pp. 293-302 and 633-682.
  QUALITY CONTROL -- Water used to fill BOD bottles SHOULD come from the same grab sample.
  QUALITY CONTROL -- Incubate the samples for up to 4 hours.
  QUALITY CONTROL -- There should be at least 1,000 cpm (counts per minute) in the filtered sample for statistical significance (Strickland and Parsons, 1968).

- Cell Volume of Phytoplankton: Determine the shape of a cell and then the volume of a cell by using the simplest geometric configuration. Calculate the total volume of any phytoplankton species by multiplying the average cell volume in cubic micrometers by the number per liter.
  QUALITY CONTROL -- For better representation of cell volume, measure 20 individuals of each species to get average cell volume for each sampling period.
  QUALITY CONTROL -- Be exact on the subsample volume from the well-mixed sample.
  QUALITY CONTROL -- Keep a consistent counting system.

- Cell Surface Area of Phytoplankton: Same as above, but measure the cell surface area instead.
  QUALITY CONTROL -- Same as described for cell volume of phytoplankton.

TABLE 3.2.6 THE PRESERVATION OF ZOOPLANKTON (U.S. EPA, 1973)

Preservatives	Preparation	Usage
Formalin	Add sodium tetraborate to obtain pH of 7.0 to 7.3. Obtain a final concentration of 5% neutral Formalin.	Preserve grab samples.
Formalin plus glycerin	Add 5% glycerin to 5% neutral Formalin.	Preserve the concentrated net samples.
Ethanol plus glycerin	Add 5% glycerin to 70% ethanol.	Preserve the concentrated net samples.
Rose Bengal stain	Add 0.04% Rose Bengal stain to 5% neutral Formalin.	Differentiate animal and vegetative material in turbid samples
Freezing	The concentrated sample is placed in a fine-meshed bag, drained of excess water, placed in a plastic bag, and frozen for laboratory processing.	For chemical analysis of zooplankton samples.

EXAMPLE: ZOOPLANKTON

Sample Collection

- Sampling equipment: a messenger-operated water bottle, or metered plankton net is often used for collecting quantitative samples. Filter surface-water samples through nylon netting or tow an unmetered plankton net through the water to obtain semi-quantitative samples. Towing from an outboard motor boat and casting of nets are two common techniques in sampling. Tows can be vertical, horizontal or oblique tow for different purpose of study. Net casting is used to obtain a qualitative estimate of relative abundance and species present. To sample most sizes of zooplankton, two nets of different mesh size can be attached a short distance apart on the same line.
  QUALITY CONTROL -- When towing with a boat is employed, maintain speed to ensure a wide angle (near 60°) for easy calculation of the actual sampling depth of the net.
  QUALITY CONTROL -- Clean nylon nets thoroughly, rinse with clean water and dry before storing.

QUALITY CONTROL -- Rinse messenger-operated samplers with clean water, dry and lubricate all moving parts with light machine oil.

- Sample Volume: In lakes, large rivers, estuaries and coastal waters, filter 1.5m^3 (horizontal tow) to 5m^3 (oblique tow) of water through nets for adequate representation of species present. For samples in flowing streams and ponds, filter 20 liter surface water through a No. 20 net to obtain an estimate of zooplankton present.
  QUALITY CONTROL -- Be sure to obtain the exact volume of sample for quantitative analysis.

- Sample Preservation: Preserve zooplankton samples with 70% ethanol, 5% neutral Formalin (pH of 7.0 to 7.3), or Lugol's solution (Rand et al, 1975). Freeze the concentrated samples for chemical analysis (U.S. EPA, 1973). See Table 3.2.6 for the detailed description of zooplankton preservation.
  QUALITY CONTROL -- Usually, use Formalin to preserve samples obtained from coastal waters.
  QUALITY CONTROL -- If the sample is taken from estuarine or sea water, the nylon bag (used to hold concentrated net samples for chemical analysis) must be dipped several times in distilled water to remove the chloride from interstitial seawater, as chloride can interfere with carbon analysis.

Sample Preparation

- Concentrate zooplankton samples by sedimentation and then mount them on slides as directed in Standard Methods, 14th Edition (page 1020) if desired.
  QUALITY CONTROL -- Must recover organisms (especially cladocera) that cling to the surface of the water in the settling tube.

Sample Analysis

- Qualitative Analysis: Make an initial examination. Identify the small (nanno) zooplankton during the routine phytoplankton qualitative analysis. Identify Copepoda, Cladocera and other larger forms with the use of a binocular dissecting microscope at a magnification of 20 to 40. Identify rotifers at 100. All animals should be identified to species if possible.
  QUALITY CONTROL -- Use all available, appropriate taxonomic reference at the bench. See a list of recommended references (U.S. EPA, 1973).
  QUALITY CONTROL -- Use taxonomic expertise in identification of questionable specimens.

- Quantitative Analysis -- Pipet Method: Dilute the concentrated sample. Withdraw 1 ml of subsample from the center of well-agitated water-plankton mixture with a 1-ml Stempel pipet. Transfer the subsample to a gridded culture dish (110 x 15 mm) with 5-mm squares. Enumerate (about 200 zooplankters) and identify under a dissecting microscope (U.S. EPA, 1973).
  QUALITY CONTROL -- Randomly select 10 strips for rotifer count.

QUALITY CONTROL -- Accurately determine the volume of the counting chamber from its inside dimensions because this volume changes the outcome of the calculated count.

- Biomass Determination -- Dry and Ash-free Weight: Determine dry weight by placing the aliquot of concentrated sample in a tared porcelain crucible and drying at 105°C for 24 hours. Subtract the weight of the crucible to obtain the dry weight. After the dry weight is determined, place the crucible in a muffle furnace at 500°C for 1 hour. Cool, wet the ash with distilled water, and bring to a constant weight at 105°C. Subtract the weight of crucible and ash from the dry weight to obtain ash-free weight. This method is sometimes used for phytoplankton biomass determination.
  QUALITY CONTROL -- Wash the sample well with distilled water by settling to reduce the amount of contamination.
  QUALITY CONTROL -- Must collect sufficient sample to provide several aliquots each having 100 mg wet weight or 10 mg dry weight because at least two replicate aliquots must be processed for each sample. Must keep the temperature in the oven or furnace constant for all drying processes.

- In addition to the aforementioned techniques for biomass determination, there is a recently developed method of measuring adenosine triphosphate (ATP) in plankton that provides a means of determining the total viable plankton biomass. According to Weber (1973), the ratio of ATP to biomass varies somewhat from species to species, but appears to be constant enough to permit reliable estimates of biomass from ATP measurements. The method is simple and relatively inexpensive. The instrumentation is stable and reliable. The method also has many potential applications in entrainment and bioassay research, especially plankton mortality studies. See equipment and reagents used, procedure, and calculation of ATP in Standard Methods, 14th Edition (Rand et al., 1975).

- Moreover, the "nitrogen fixation" idea is introduced by aquatic physiologists to measure metabolic rates of plankton communities in the water. The two methods for estimating nitrogen fixation rates in the laboratory are the ^{15}N isotope tracer method and the acetylene reduction method. It is found that the great variation in the rate of nitrogen fixation with different types of organisms and with the concentration of combined nitrogen in the water makes it impossible to use nitrogen fixation rates to estimate biomass of nitrogen-fixing organisms in surface waters. But the acetylene reduction method is useful in measuring nitrogen budgets and in algal assay work (Stewart et al., 1967 and 1970; Weber, 1973).

3.2.3.2 Periphyton--

Periphyton is also known as "Aufwuchs" in German, which can be seen in some literature. It is defined as "a community of microscopic plants and animals associated with the surface of submersed objects. Many of the protozoa and other minute invertebrates and algae that are found in the plankton also occur in the periphyton" (Rand et al., 1975).

Two types of sampling are generally used for periphyton sample collection: qualitative and quantitative sampling. Qualitative studies concerned only with systematics of periphyton require no elaborate or complicated apparatus for the collection of samples. Knives, scrapers, and similar implements have sometimes been modified for specific habitats, e.g., a curved knife for scraping epiphytic periphyton from bulrushes (Wetzel and Westlake, 1974). For the measurement of biomass, artificial substrate is a most widely accepted sampling method compared to those devices that have been developed for the collection of quantitative samples from irregular surfaces.

Since the periphyton community is an excellent indicator of water quality, the selection of a minimum of two sampling stations will be required to provide data on the community in both the pollution-free zone and the polluted zone in a body of water. However, a more intensive sampling program is recommended if possible.

EXAMPLE: PERIPHYTON

Sample Collection

- Natural substrate method: qualitative samples may be taken by scraping submerged rocks, sticks, and other substrates available at the station. QUALITY CONTROL -- This method is not recommended for the collection of quantitative samples because of inaccurate measurements of sampling areas.

- Artificial substrate method: The standard (plain, 25 x 75 mm) glass microscope slide is a most suitable artificial substrate for quantitative sampling. Plexiglas slides may be used in place of glass slides. In large rivers or lakes, a floating sampler (Rand et al., 1975, p. 1046) is advantageous when turbidities are high and the substrates must be exposed near the surface. In small, shallow streams or littoral areas of lakes where turbidity is not a critical factor, substrates may be exposed in two possible ways: (a) attach the substrates with PLASTIC TAK adhesive to bricks or flat rocks in the stream bed, or (b) anchor Plexiglas racks to the bottom to hold the substrates. In areas where the siltation is a problem, hold the substrate in a vertical position to avoid a covering of silt (U.S. EPA, 1973).
  QUALITY CONTROL -- The depth of exposure must be consistent for all sampling sites.
  QUALITY CONTROL -- Because of unexpected fluctuations in water levels, currents, wave action, and the threat of vandalism, duplicate samplers should be used (U.S. EPA, 1973).
  QUALITY CONTROL -- A minimum of four replicate substrates should be taken for each type of analysis (U.S. EPA 1973).

- After taking samples, further separations may be needed to obtain the different components of periphytes (e.g., algae, diatom) relatively free from detritus and mineral matter. Sample preparation varies according to the method of analysis; see the 14th edition of Standard Methods, Section

1003C (Rand et al., 1975). Generally, preserve samples that are taken for counting and identification in 5% Formalin or other suitable material. Wetzel and Westlake (1974) suggest that Lugol's iodine (made up of 10 g of pure iodine, 20 g of KI, 200 ml of distilled water and 20 g of glacial acetic acid combined a few days prior to using; store the solution in dark glass bottles; added to the samples in a 1:100 ratio) and 5% mercuric chloride are particularly suitable. If the material is for chlorophyll analysis, store it at 4°C in the dark in 100 ml of 90% aqueous acetone. Use bottle caps with a cone-shaped polyethylene seal to prevent evaporation. Sladeckova (1962) gives detailed suggestions for the collection, preservation, and transport of periphyton on artificial substrates.

Sample Analysis

- Identification
  QUALITY CONTROL -- Use all available taxonomic references for each possible composition of periphyton community: algae, fungi, protozoae, rotifer, microcrustacea.
  QUALITY CONTROL -- Consult the taxonomic authority whenever necessary.

- Counting: Sedwick-Rafter count is a universal method. The quantitative determination of organisms on a substrate can be expressed as:

$$\text{No. cells/mm}^2 = \frac{C \times 1000\ \text{mm}^3 \times V \times DF}{L \times W \times D \times S \times A}$$

where C = number of cells counted (tally)
V = sample volume, ml
DF = dilution factor
L = length of strip, mm
W = width of strip (Whipple grid image width), mm
D = depth of a strip (S-R cell depth), mm
S = number of strips counted
A = area of substract scraped, mm^2

QUALITY CONTROL -- Thorough mixing must be done by vigorous shaking prior to counting.
QUALITY CONTROL -- If a material is too concentrated for a direct count, a proper dilution must be made.
QUALITY CONTROL -- Avoid clumps of cells in the counting cell because these clumps could result in inaccuracy of the count.

- Diatom proportional count: Mount diatom slides as described in Standard Methods (see Plankton, 1002 D.3) or "Biological Field and Laboratory Methods" (U.S. EPA, 1973, page 11 in Plankton Section). Identify and count all diatoms within the borders of the grid until 250 cells (500 halves) are tallied.
  QUALITY CONTROL -- The slides must be labelled with all relevant information.
  QUALITY CONTROL -- Use "A Guide to the Common Diatoms at Water Pollution Surveillance System Stations" (Weber, 1971) as a basic reference.

3.2.3.2 Macrophyton--

Macrophytes are all aquatic plants possessing a multi-cellular structure with cells differentiated into specialized tissues. Their communities range from completely submerged stands of large algae (e.g., *Chara*, *Cladophora*), mosses (e.g., *Fontinalis*), pteridophytes (e.g., *Isoetes*) and angiosperms

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(e.g., Elodea, Ranunculus ssp.), through stands of rooted plants with floating leaves (e.g., Nymphaea) and mats of floating plants with emergent leaves (e.g., Eichhornia, Lemna) to wetlands with plants with little except their underground parts submerged (e.g., Equisetum, Phragmites, Rhizophora).

As usual, there are two types of studies in relation to macrophyton: qualitative and quantitative sampling. Before beginning a quantitative investigation it is desirable to have a statistical design which will assist in determining the best sampling procedure, sampling area size, and number of samples. It is recommended that the appropriate TP (Terrestrial Productivity) techniques should be adopted (Milner and Hughes, 1968; Blackburn et al., 1968; Edwards and Owens, 1960; Forsberg, 1959; Jervis, 1969; Westlake, 1966; Westlake, 1968).

Due to natural phenomena, there are frequent shifts in plant population of a particular site or location. Quality control is generally obtained by standardizing the time of the year and accumulating data over a long period of time. The specific quality control in sample collection, sample preparation and sample analysis will be summarized and briefly discussed below.

TABLE 3.2.7 SAMPLING EQUIPMENT FOR MACROPHYTES
(Westlake, 1974)

Type of Equipment	Suggested Application
Scoop, diver operated	Important root systems
Ekman dredge	Mud; small root system
Petersen dredge	Hard bottom; poor sampling
Petersen dredge, modified	Hard bottom; better sampling
Cylindrical sampler	Soft bottom; upright plants, small root system
Quadrate frame sampler	Soft bottom; tall plants, small root systems
Pronged grab	Luxuriant vegetation; roots from soft bottom

EXAMPLE: MACROPHYTON

Sample Collection

- Selection of sampling site and frequency: The general aim will be to

remove and weigh the vegetation from enough known areas to obtain a mean biomass sufficiently accurate to show significant differences between sampling periods and sites (Westlake, 1974).
QUALITY CONTROL -- Individual plants should be collected at each sampling site sufficient to establish the frequency and diversity of the population. At least four sites should be selected for each location (U.S. Department of the Interior, 1972).
QUALITY CONTROL -- Normal statistical methods must be applied with caution because the spatial variation is often nonrandom (Westlake, 1974).
QUALITY CONTROL -- Select the size and shape of the sampling area to reduce the variability, e.g., large quadrates, rectangular quadrates in contagious (clumped) communities, summed quadrates along transects parallel to gradients (Westlake, 1974).
QUALITY CONTROL -- In stands of limited area care must be taken to avoid damaging the community excessively and affecting subsequent samples (Westlake, 1974).
QUALITY CONTROL -- Avoid sampling or experiments in previously disturbed areas (Westlake, 1974).

- Sampling equipment: See Table 3.2.7.
  QUALITY CONTROL -- Select appropriate gear for personnel and nature of the survey, types of plants.

- Sampling techniques: Approach the sampling areas by wading, in boats or by diving; remove plants by hand or by sampler.
  QUALITY CONTROL -- Collect base maps and detail information related to terrain concerning the safety of personnel.
  QUALITY CONTROL -- Use appropriate mode of transportation related to the area.
  QUALITY CONTROL -- Mark off areas for hand sampling with stakes and strings if large, or quadrate frames if smaller, to avoid overlap.
  QUALITY CONTROL -- Use a net set downstream of the sampling area to collect the cut submerged plants.

Sample Preparation for Macrophytes (including washing, sorting, sub-sampling, and drying for future analysis)

- Wash in a shallow sloping trough with a jet of water (approx. 2.5 atm.) to remove soil, epiphytes, and animals.
  QUALITY CONTROL -- Be sure to wash well because the total weight of unwanted material may exceed the weight of the plants.
  QUALITY CONTROL -- Recover plant fragments by flotation by passing the water through a 1/2-inch (approx. 12.7-mm) mesh net.

- Sort into different species for productivity studies.
  QUALITY CONTROL -- Requires trained and experienced personnel but no special equipment is needed.

- Preservation.
  QUALITY CONTROL -- Small, delicate samples should be preserved in buffered 4% Formalin solution.

QUALITY CONTROL -- All other samples may be dried in a plant press and mounted for further identification.

- Record and label.
  QUALITY CONTROL -- All necessary information must be recorded on the label and field notebook (or data sheet).

- Dry: Use a domestic spin-drier and/or general purpose laboratory oven.
  QUALITY CONTROL -- Dried samples must be cooled in a desiccator and sealed in polyethylene bags before weighing, as many samples can take up to 10% moisture from air.

- Subsample.
  QUALITY CONTROL -- Random sampling process should be used.
  QUALITY CONTROL -- The weed should be chopped and well mixed before taking subsamples.

- Individual specimens should be properly prepared (mounted or preserved) and annotated with recorded data before the sample analysis begins. Sample analysis of macrophytes includes, in general, identification, biomass (or standing crop) and productivity. Dryweight biomass measurement may be summarized as follows: A sample is taken from a small defined area with conspicuous borders. The wet weight of material is obtained after the plants have drained for a standard period of time. The sample is then dried for 24 hours at 10^{0}C and reweighed. The dry weight of vegetation per unit area is then calculated.

Sample Analysis

- Identification: Identify samples according to family, genus, and species.
  QUALITY CONTROL -- Use appropriate taxonomic texts for identification. See reference list relevant to aquatic plants in Section 3.2.4.2.

- Biomass or standing crop: See description of method just above.
  QUALITY CONTROL --Balances capable of holding bulky samples, weighing up to 5 to 10 kg of fresh weights, will be needed for samples from 1 square meter.
  QUALITY CONTROL -- Balances capable of weighing up to 1 kg are most convenient for dry weight determination.
  QUALITY CONTROL -- For consistent results, the oven must be calibrated to 105^{0}C.

- Productivity: Use of isolated shoots for emergent macrophytes.
  QUALITY CONTROL -- Never use this method for productivity of benthic plants.
  QUALITY CONTROL -- The water used for incubation needs to be taken from the same location as the plants because of the stratification of nutrients, temperature, etc., in many habitats.

- Oxygen exchanges in light and dark enclosures in situ for submerged macrophytes.
  QUALITY CONTROL -- Possible sources of error in the application of the oxygen techniques are lacunal storage of oxygen, and irregular utilization of oxygen for respiration due to intermittent current stirring.
  QUALITY CONTROL -- The results must be interpreted with extreme caution.

- ^{14}C technique in situ for submerged macrophytes.
  QUALITY CONTROL -- The incubation chambers are recommended to be cylinders made of clear Plexiglass in various sizes to permit placement in situ around different species of plants.
  QUALITY CONTROL -- The volume of the chamber must be calibrated.
  QUALITY CONTROL -- The rooted organs of macrophytes must be included in the chambers.
  QUALITY CONTROL -- Keep the incubation to a short mid-day period (e.g., from 10:00 to 14:00 hr) of four hours because evidence suggests that the production rates of this mid-day increment are good mean values under a majority of light and other environmental conditions.
  QUALITY CONTROL -- The excretion of organic matter, i.e., carbohydrates, during the photosynthesis by macrophytes presents a possible source of error in the employment of the ^{14}C techniques.

- Chlorophyll determinations: Analyze for chlorophyll a, b, c, and d.
  QUALITY CONTROL -- Ensure thorough acetone extraction by grinding or homogenizing material.
  QUALITY CONTROL -- Spectrophotometer must be adjusted and calibrated according to manufacturer's manual at regular time intervals.

3.2.3.4 Macroinvertebrates--

The macroinvertebrates, as discussed in this section, are animals that are large enough to be seen by the unaided eye and can be retained by a U.S. Standard Number 30 sieve (28 meshes per inch, 0.595 mm opening). Many small or slender individuals and early life stages of these invertebrates will pass through the sieve and not be included. The sieve, however, is a practical and rapid method of sorting most macroinvertebrates from their substrate. They may be collected by various methods using equipment such as grabs (or dredges), Surber samplers, corers, nets, seines, artificial substrates, trawls, or other specialized samplers. A few basic requirements for field invertebrate sampling are:

- The selection of the best sampler requires evaluation of the physical conditions in which the sampler will be used. These conditions include substrate type, and depth
- The kind of sampler selected is used consistently for a particular area so that population characteristics may be compared
- Use more than one sampler type to obtain good representation of the fauna which reside in natural substrates

EXAMPLE: MACROINVERTEBRATES

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Sampling Equipment/Methods

- Grab devices.
  QUALITY CONTROL -- Understand the patchy distribution of some organisms in nature.
  QUALITY CONTROL -- Use grab sampling only for qualitative studies, i.e., estimate of numbers of taxa. Due to the problems in depth of penetration, angle of closure, completeness of closure of the jaws and loss of sample material during retrieval, creation of a "shock" wave and consequent "wash-out" of near-surface organisms, and stability of the sampler at the high-flow velocities often encountered in rivers, grab-collected samples provide an imprecise estimate of aquatic macroinvertebrate populations (U.S. EPA, 1973).
  QUALITY CONTROL -- Collect additional samples to increase precision in the selected method.

- Sieving devices.
  QUALITY CONTROL -- Collect the samples from downstream to upstream.
  QUALITY CONTROL -- Stand on the downstream side of a sieving device and take replicates in an upstream or lateral direction.

- Coring devices.
  QUALITY CONTROL -- Best suitable for sampling the relatively homogeneous soft sediments of the deeper portions of lakes.

- Nets.
  QUALITY CONTROL -- In the aquatic environment, place the top of the drift nets just below the surface to lessen the chance for collection of floating terrestrial insects.
  QUALITY CONTROL -- For field insects study, use sweep-net method to compare populations from one area at different times, or from different areas. Bear in mind that three major difficulties encountered in sampling are: daily changes in the environment, differences in the growth habits and structure of the vegetation, and differences in the agility and tenacity of the insects (Davis and Gray, 1966).

- Artificial substrates.
  QUALITY CONTROL -- Use EPA-recommended samplers (multiple-plate sampler and rock basket sampler) for studying a macroinvertebrate community.
  QUALITY CONTROL -- Caution should be exercised in the reuse of samplers that may have been subjected to contamination by toxicants, oils, etc.
  QUALITY CONTROL -- Adoption of a 6-week exposure period is provisionally recommended as standard (Rand et al., 1975).
  QUALITY CONTROL -- Unless the water is exceptionally turbid, a 1.2-meter (4-foot) depth is recommended as standard.
  QUALITY CONTROL -- Never use artificial substrates to measure the productivity of a particular environment.

Sample Preparation

- Sieving.
  QUALITY CONTROL -- Use a U.S. Standard No. 30 sieve to separate samples collected with conventional sampling devices.
  QUALITY CONTROL -- Sieving should be done in the field immediately after sample collection.

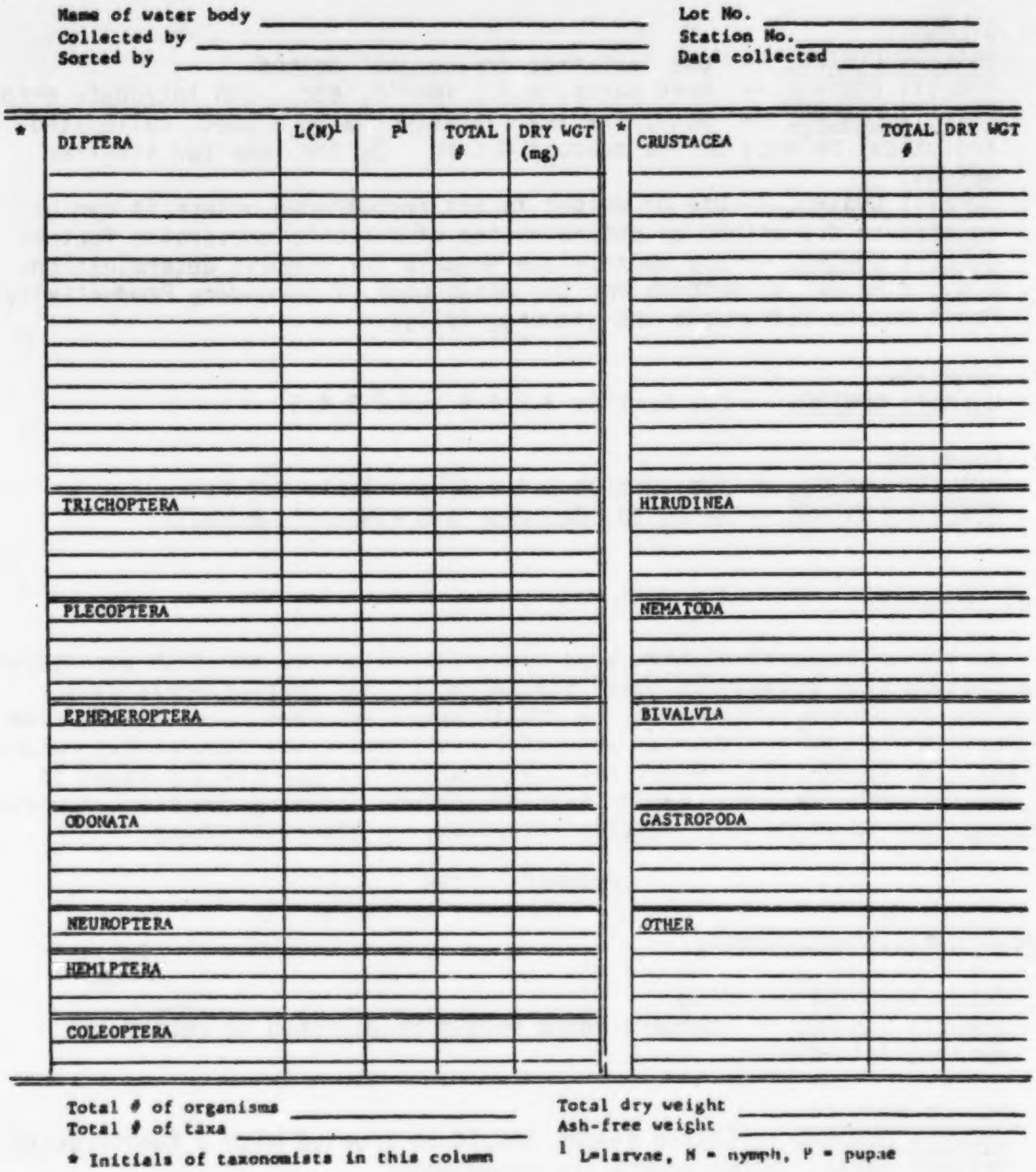
- Preservation.
  QUALITY CONTROL -- Preserve the samples in 70 percent ethanol. Do not use Formalin. When necessary, specimens could be transferred from alcohol to pins.
  QUALITY CONTROL -- Samples are preserved immediately in plastic or glass containers.
  QUALITY CONTROL -- Rose Bengal stain at a concentration of approximately 200 mg/l in the preservative may be used to stain the animals to aid in sorting (Rand et al., 1975; Slack et al., 1973).

- Records and labelling.
  QUALITY CONTROL -- Write all information (see Section 4.2.1.6) on water-resistant labels with a waterproof marker.
  QUALITY CONTROL -- This information must be recorded in a permanent record.

- Sorting and subsampling.
  QUALITY CONTROL -- Subsampling may be used for samples containing excessively large numbers of organisms before sorting. But be sure that sample is thoroughly mixed and distributed evenly over the bottom of a shallow tray before delineation.
  QUALITY CONTROL -- All organisms should be sorted into major categories (i.e., insect orders, molluscs, worms) and placed in vials containing 70 percent ethanol.
  QUALITY CONTROL -- All vials from a sample should be labelled internally with the sorter's name and the sample identification (log) number and kept as a unit in a suitable container until organisms are identified, counted and the data are recorded on the bench sheets. See a typical laboratory bench sheet in Table 3.2.8.
  QUALITY CONTROL -- A check on the sorting procedure can be done by re-examination of the sample or by aliquot analysis.

Sample Analysis

- Identification.
  QUALITY CONTROL -- The accuracy of identification will depend greatly on the available taxonomic literature. See Section 3.2.4.2.
  QUALITY CONTROL -- Store identified specimens in a reference collection for quality control checks.
  QUALITY CONTROL -- Mount the whole organisms or parts thereof on glass slides for examination at high magnification to make species identification whenever necessary. Make proper labelling on the prepared slides.
  QUALITY CONTROL -- Rear the collected insect larvae in the laboratory to aid in identifying the difficult-to-identify species.



Name of water body ______________________ Lot No. ____________

Collected by ______________________ Station No. ____________

Sorted by ______________________ Date collected ____________

*	DIPTERA	L(N)[1]	P[1]	TOTAL #	DRY WGT (mg)	*	CRUSTACEA	TOTAL #	DRY WGT
	TRICHOPTERA						HIRUDINEA		
	PLECOPTERA						NEMATODA		
	EPHEMEROPTERA						BIVALVIA		
	ODONATA						GASTROPODA		
	NEUROPTERA						OTHER		
	HEMIPTERA								
	COLEOPTERA								

Total # of organisms ____________ Total dry weight ____________

Total # of taxa ____________ Ash-free weight ____________

* Initials of taxonomists in this column

[1] L=larvae, N = nymph, P = pupae

(Weber, 1973a)

Figure 3.2.3 Laboratory bench sheet for aquatic macroinvertebrates (Weber, 1973a).

QUALITY CONTROL -- Identification can be checked by re-examination or by multiple analysis.

- Biomass.
  QUALITY CONTROL -- Use "ash-free dry weight" method.
  QUALITY CONTROL -- Hard parts, e.g., shells, etc., can introduce errors.
  QUALITY CONTROL -- Determine the wet weight with a good, calibrated analytical balance to the nearest 0.1 mg. Do the same for ash-free weight.
  QUALITY CONTROL -- Use of weight is not recommended unless it can be equated to dry weight by determination of suitable conversion factor.
  QUALITY CONTROL -- Use appropriate manuals for biomass determination, e.g., a Manual on Methods for the Assessment of Secondary Productivity in Fresh Waters (Edmondson and Winberg, 1971).

- Bioassay.
  QULAITY CONTROL -- See Section 3.2.4.4 and 3.2.4.5.

- Counting.
  QUALITY CONTROL -- See Section 3.2.4.3, and Table 3.2.9.
  QUALITY CONTROL -- Refer to Edmondson and Winberg's manual.

3.2.3.5 Fish--

Many sampling methods have been available to assess the fish populations. The methods vary greatly in their precision and the cost-effectiveness required to obtain information. A creel census or other catch record from commercial and sport fisheries is useful for showing the harvestable nature of the fish population. Other methods in which all species and sizes of fishes in a body of water may be sampled include draining the body of water, seining, use of chemicals, netting, trapping, or electroshocking.

EXAMPLE: FISH

Sampling Equipment/Methods

- Catch records/recording.
  QUALITY CONTROL -- Standard forms should be designed to record the desired information.

- Seines/seining.
  QUALITY CONTROL -- Cotton seines should be treated with a fungicide to prevent decay. Nylon seines are recommended.
  QUALITY CONTROL -- Seining is only effective in shallow water and is more useful in qualitative study.

- Nets/netting (gill nets, trammel nets, etc.).
  QUALITY CONTROL -- Gill nets made of multifilament or monofilament nylon are recommended.
  QUALITY CONTROL -- Replace the individual floats (usually supplied with nets) with a float line made with a core of expanded foam and use a lead

core leadline instead of individual lead weights to reduce net entanglement problems.
QUALITY CONTROL -- Gill and trammel netting are in extensive use to sample fish populations in estuaries, lakes, reservoirs and large rivers. When drifting gill or trammel nets are set, they require constant surveillance.

- Traps/Trapping (Trap nets, hoop nets, fyke net, etc.).
  QUALITY CONTROL -- Trap and hoop nets made of nylon have a longer life. Protect cotton nets from decay by treatment.

- Trawls/trawling (fry trawl, otter trawl, etc.).
  QUALITY CONTROL -- The use of trawls requires experienced personnel.
  QUALITY CONTROL -- Trawls are best used to gain information on a particular species of fish rather than to estimate the overall fish population.

- Chemicals/chemical fishing (rotenone, antimycin, etc.).
  QUALITY CONTROL -- The most widely used toxicant is rotenone. Recommended concentrations of the 5% preparation: 0.1 ppm for sensitive species, 0.5 ppm for most species, and 1 to 2 ppm for resistant species.
  QUALITY CONTROL -- Chemical sampling is usually employed on a spot basis, e.g., on embayment of a reservoir or a short reach of a river.
  QUALITY CONTROL -- An appropriate efficient spraying equipment must be selected to apply rotenone emulsion.

- Electroshocker (AC, DC, etc.).
  QUALITY CONTROL -- Before deciding which design to use, the biologist should carefully review the literature. See more than 30 listed references in "Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents" (U.S. EPA, 1973).
  QUALITY CONTROL -- The crew should wear rubber boots and electrician's gloves and adhere strictly to safety precautions.
  QUALITY CONTROL -- Electrofishing is more effective and efficient for sampling fish population at night.
  QUALITY CONTROL -- Electrofishing devices are effective in collecting most sizes and species of fish from many different environments.

- Fish studies are usually dependent on data collected in the field and include fish identification, weight, length and other observations. The collected samples should be prepared as described in the following for further studies, e.g., age, growth and condition of fish, and fish kill.

Sample Preparation

- Preservation and storage.
  QUALITY CONTROL -- A 10% Formalin is usually used as a fish preservative. Preserve fish in the field. Add 3 to 5 g borax and 50 ml glycerin per liter of Formalin.
  QUALITY CONTROL -- Fish longer than 75 mm should be slit on the right side of body to allow penetration of the preservative.

QUALITY CONTROL -- For permanent preservation, specimens must be washed in running water for at least 24 hours and placed in 40 percent isopropyl alcohol.
QUALITY CONTROL -- Only plastic or glass containers should be used.
QUALITY CONTROL -- Samples may also be iced or placed in dry ice for preservation.

- Data Recording.
  QUALITY CONTROL -- See Section 3.2.1.4.
  QUALITY CONTROL -- Use the metric system for length and weight measurements.

- Sample analysis is usually done in the laboratory and after preservation and includes identification, age, and growth determination, condition factor, histopathology, and flesh-tainting.

Sample Analysis

- Identification.
  QUALITY CONTROL -- Use appropriate manuals for fish identification. See reference list in (U.S. EPA, 1973).
  QUALITY CONTROL -- Confirm questionable identification with Federal, state and university fish taxonomists.

- Age and growth.
  QUALITY CONTROL -- Use appropriate personnel for age determination.
  QUALITY CONTROL -- Use adequate handbook for the age and growth study. For example, Carlander's Handbook is good for freshwater fishes (Carlander, 1969).
  QUALITY CONTROL -- Use available written computer package for the back calculation of fishes' growth history.

- Condition (including natural and man-induced mortalities).
  QUALITY CONTROL -- Use trained and experienced personnel.
  QUALITY CONTROL -- The speed of response to fish kill is a key to success.

- Counting.
  QUALITY CONTROL -- See Section 3.2.4.3, and Table 3.2.8.
  QUALITY CONTROL -- Use adequate handbook for fish population study, e.g., Ricker's handbook is good for fish in freshwater (Ricker, 1971).

- Flesh tainting.
  QUALITY CONTROL -- Uniform taste quality should be assured before exposure of test fish.
  QUALITY CONTROL -- A test panel should be trained in flesh tainting and should be given acceptable samples for comparison.

- Bioassays.
  QUALITY CONTROL -- See Sections 3.2.4.4 and 3.2.4.5.

- Biomass.
  QUALITY CONTROL -- See Section 3.2.4.6.

QUALITY CONTROL -- Use Ricker's Handbook (Ricker, 1971).

3.2.3.6 Birds--

EXAMPLE: BIRDS

Sample Collection

- Qualitative study: Both shooting and trapping techniques are used by bird collectors for collecting qualitative specimens. A shotgun armed with different-sized shots (e.g., Nos. 10, 6, 4, 2 and BB) is necessary for general collecting of birds. Various traps are indicated in Table 3.2.1.
  QUALITY CONTROL -- Use proper shooting equipment. Never use a rifle to collect birds as the rifle bullet tears them all up.

- Qualitative study: Trapping is only the means for a catch-mark-recapture (CMR) study for estimating avian population. Sampling plans which are very critical in the quantitative study of birds should include site selection of sampling, the frequency of sampling, number of sampling units and size of sampling plots. The size of the sampling unit (or plot) depends on the size, mobility and abundance of the species. For partridge, 100 hectares may be recommended (Petrusewicz and Macfadyen 1970). The number of sample units depends on the homogeneity of the habitat as well as on the numbers and characters of the distribution of birds in it. In a normal heterogeneous habitat, an average of 5 to 10 sampling units is usually adequately representative. In an unknown habitat a larger number is recommended (Petrusewicz and Macfadyen, 1970).
  QUALITY CONTROL -- The project supervisor should consult a statistician for a final decision on a formal sampling plan. The complete review of historical information on areas and species studies would be greatly helpful.
  QUALITY CONTROL -- Use appropriate means for catch-mark-recapture (CMR) study.
  QUALITY CONTROL -- Trap sites, marked birds and other pertinent information should be recorded permanently. All entries should be in carbon ink.

Sample Preparation

- Skinning and Mounting: Anderson (1964) has discussed these techniques in Chapter IV, Collecting and Skinning Birds of his book entitled, "Methods of Collecting and Preserving Vertebrate Animals."
  QUALITY CONTROL -- No samples or specimens will be analyzed without proper identification labels.
  QUALITY CONTROL -- There must be a capture sheet for every bird.
  QUALITY CONTROL -- Avoid the use of abbreviations and laboratory jargon; in ten years or less they may be difficult to be understood.

- Preservation: Alcohol and formaldehyde are two commonly used liquid preservatives for preserving soft parts of birds, stomach contents, and bird droppings. A mixture of powdered arsenic and powdered arsenic plus borax (in about equal proportions by volume) is the most satisfactory preservative for the birds' skins.
  QUALITY CONTROL -- If laparotomy is carried out, laparotomy sheets including sex, band number, the date and time of laparotomy, name of operation, etc., must be completed and filed permanently.

- Labelling: Label all prepared specimens with the pertinent information, e.g., identification number, location and date of collection, etc., in accordance with the pertinent record.

Sample Storage

- Deepfreeze or refrigerate the samples which are delayed for preparation or analyses.
  QUALITY CONTROL -- No samples should be delayed for further analyses, e.g.,chemical residue analysis in the laboratory.

- Fumigate the skinned and stuffed birds for long-term storage. DDT or moth balls can be used as fumigants.
  QUALITY CONTROL -- All skinned, or preserved specimens should be stored with labels for permanent records.

Sample Analysis

- Identification: Identify all specimens to species level whenever possible.
  QUALITY CONTROL -- Be exact in identification with available taxonomic references. Refer to an authority for identification of questionable birds.

- Number of Birds: The methods of studying bird populations are greatly varied depending on the species studied, the habitat, technical means, time and money available. Table 3.2.8 shows the various methods that have been used by wildlife biologists. Two of the most familiar methods are direct count and mark-and-recapture study.
  QUALITY CONTROL -- Good eyes of the individual making count, and a good pair of binoculars are essential in estimating bird population.
  QUALITY CONTROL -- If a sample census is used, a census datum should be accompanied by a clear statement of constraints and definitions under which it was collected and by a critical evaluation of its accuracy.

- Weight and Biomass: Obtain individual bird weight by weighing a representative number of birds and calculating average (X). Measure biomass by adding up the weights of all birds or calculate by multiplying the average (X) by numbers estimated (N).
  QUALITY CONTROL - Choose the individuals that represent either classes or a succession of known time intervals in the history of their population.

QUALITY CONTROL -- The accuracy of biomass measurement is dependent completely on the accuracy of determinations of numbers and of weighing.

- Bioassay. See Section 3.2.4.4 and 3.2.4.5.

3.2.3.7 Mammals--

EXAMPLE: MAMMALS

Sample Collection

- Qualitative study: The larger mammals are almost invariably taken by shooting, and some of the smaller species, such as rabbits and squirrels, are more often shot than trapped. A shotgun is indispensable for general collecting of mammals, too. A double-barrelled gun is preferable, and shells loaded with different size shots, Nos. 10, 6, 4, 2, and BB.
  QUALITY CONTROL -- Use appropriate shooting equipment for collecting mammal specimens. For example, the rifle is not ideal for collecting the smaller mammals as the rifle bullet tears them up too much.

- Quantitative study: Trapping is more often used for mark-and-recapture studies to estimate animal population. Traps used by collectors vary with animals to be trapped and collecting individuals. See Table 3.2.1. Sampling plans are a must in the quantitative study of mammals. The plans, as usual, include sampling frequency, sampling site selection, number of sampling units and size of sampling plots. The first two elements depend heavily on the objective of the study. The size of the sample unit depends on the size, mobility, and abundance of the species. For small mammals, 2 to 6 hectares may be recommended, and for hares and deer, 100 hectares (Petrusewicz and Macfadyen, 1970). The number of sample units depends mainly on the homogeneity of the habitat as well as on the numbers and character of the distribution of animals in it. In a habitat of normal heterogeneity, an average of 5 to 10 sampling units is usually adequately representative. In an unknown habitat a larger number is recommended (Petrusewicz and MacFayden, 1970).
  QUALITY CONTROL -- Trap sites and other records must be noted in permanent notebook.
  QUALITY CONTROL -- Use appropriate traps for various sizes of animals and their habitats.
  QUALITY CONTROL -- Consult statisticians to adopt a formal sampling plan.

Sample Preparation

- Sample labelling. Label all prepared samples with necessary information on waterproof paper and in waterproof ink.
  QUALITY CONTROL -- Specimens should always be fully labelled at the time they are prepared, as a specimen without an authentic record has no scientific value.

TABLE 3.2.8 THE METHODS FREQUENTLY USED BY WILDLIFE BIOLOGISTS FOR ESTIMATING NUMBER OF ANIMALS IN THE FIELD

Methods involving direct counts of animals:

- Territory-Mapping methods
- Drive counts
- Temporal censuses
- Extermination or total capture
- Sample censuses
- Pseudo sample censuses (e.g., The Kind Method, Frye's strip census, time-area counts, etc.)

Methods involving animal signs and related objects:

- Auditory index
- Pellet counts
- Miscellaneous indices (e.g., counts of leaf nests for squirrels)

Methods involving marked animals:

- Petersen or Lincoln Index
- The Schnabel Method
- Jolly's Method for multiple recapture experiments
- The Frequency of Capture Method
- Miscellaneous methods (e.g., Schumacher-Eschmeyer Method)

Methods involving "reduction" of population size and rate of "capture":

- The Graphical Solution
- The Leslie Method
- DeLury's Method

Method of selective reduction or increase (Dichotomy method or the change in composition method) (Overton, 1971):

Age and sex determinations, birth and death rates, etc. See "Criteria of Sex and Age" by Taber (1971), and "Population Analysis" by Eberhardt (1971). The former article describes explicitly the techniques of determining sex and age of birds and mammals while the latter article directs the wildlife biologists how to estimate the survival and recruitment rate, to analyze population structure, and to predict population size and trends. For these determinations, the following QUALITY CONTROL measures must be used:

All bird specimens should have the sex verified by dissection.

Use trained and experienced personnel.

Use available computer packages for analyzing complex, dynamic bird populations. See "Using Computers in Wildlife Management" by Adams, in Giles (1971).

Follow standard procedures for weighing and preserving avian gonads used by Avian Physiology Laboratory, Fish and Wildlife Service.

- Skinning and Preservation. See Chapter III, Skinning Mammals of "Methods of Collecting and Preserving Vertebrate Animals" (Anderson, 1964) for full details. The prepared skins, whole animals and any parts of animals can be preserved by recommended techniques described in Table 3.2.2. A mixture, in about equal proportions by volume, of powdered arsenic and powdered borax is the most satisfactory preservative for the skins of small mammals. Alcohol and Formalin (formaldehyde) are most commonly used for preserving entire specimens or any soft parts of animals, stomach contents, droppings, etc.
  QUALITY CONTROL -- Use proper preservatives in right concentrations for various animals or animal parts.

Sample Storage

- Fumigate the skinned and stuffed animals when storing with naphthaline flakes, moth-balls or insecticides (e.g. DDT).
  QUALITY CONTROL -- Store all specimens with labels for permanent records.

- Deepfreezing or refrigerating of some samples is recommended.

Sample Analysis

- Identification: Identify all specimens to species level whenever possible.
  QUALITY CONTROL -- Use all available references for exact identification and consult the proper authority, e.g., museum curators for unidentifiable animals.

- Number of mammals: The methods of determining population size are many and greatly varied, depending on the qualities of the species studied, the habitat, and technical means and time available. Main categories of methodology are total count, sample counts, catch-mark-recapture (CMR) methods, and many other specialized methods. See Table 3.2.8 for a list of methods that are described in details by Overton (1971).
  QUALITY CONTROL - If a "total census" is used, there is no question of variance or confidence limits in the sampling sense. If not, a census datum should be accompanied by an explicit statement of constraints and definitions under which it was collected and by a critical evaluation of its accuracy.
  QUALITY CONTROL -- Use the "census" methods in consistent ways throughout the study period.
  QUALITY CONTROL -- Personnel who count animals should have good eyes and be equipped with a good pair of binoculars.

- Weight and Biomass: Individual weights are obtained by collectively weighing a representative number of animals and calculating the average (X). Biomass can either be measured by summing up the weights of all animals or calculated by multiplying the average individual weights (X) obtained at a census by numbers (total estimated population, N).
  QUALITY CONTROL -- Individuals should be chosen to represent either age classes or a succession of known time intervals in the history of their

population.
QUALITY CONTROL -- The accuracy of biomass estimates depends principally on the accuracy of determinations of numbers and of weighing.

- Population analysis including age and sex composition, birth and death rates, and numerical abundance. Taber (1971) has described clearly and in detail the criteria of age and sex for birds and mammals and the ways of determining their sex and age. For other elements of population analysis, see Eberhardt's (1971) article, "Population Analysis" that instructs wildlife managers how to estimate the rate of survival and the rate of recruitment, to analyze population structure and finally to predict population size and trends.
  QUALITY CONTROL -- Use trained and experienced personnel.
  QUALITY CONTROL -- Use available computer packages for studying complex, dynamic wildlife populations. See Adams on "Using Computers in Wildlife Management" in Giles (1971).

- Bioassays. See Sections 3.2.4.4., 3.2.4.5., and 3.3.

3.2.3.8 Plants--

The following example which is in part derived from "Taxonomy of Vascular Plants" (Lawrence, 1951), includes sample collection, sample preparation, sample preservation and sample analysis, with respect to quality control.

EXAMPLE: PLANTS

Sample Collection

- Certain items of equipment are indispensable to plant collecting, particularly a collecting pick (for digging up rhizomes, deep-seated bulbs or corms, and the roots of most herbaceous plants), a strong knife or a machete, and a pair of pruning shears (for cutting woody material to pressing size). Besides, a garden rake or potato digger is useful in collecting submerged aquatic plants.
  QUALITY CONTROL -- Use standard collecting equipment. Most required collecting equipment is available from biological supply houses.
  QUALITY CONTROL -- Use one method consistently through the study period.

- Basically, there are three major ways to handle freshly collected plant material. The first, and most satisfactory method, is to press each plant as it is collected. Secondly, the plant materials are accumulated in a metal collecting can or vasculum. The third method, used more in the tropical rain forests than in temperate regions, is to carry collected specimens in a rucksack.
  QUALITY CONTROL -- Plants should be pressed or processed as soon as possible.

Preparation of Specimens

- Pressing: Conventionally, most presses comprise a pair of wood or metal frames, blotters, pressing papers, and straps or strong cord. The specimen to be pressed is arranged within the folded sheet of pressing paper that has been placed on a blotter, and another sheet is placed over it. If the plants are to be dried with aid of artificial heat, a sheet of corrugated material (ventilator) is used between each pressing paper and its specimen, otherwise no corrugates are used and the press is built up by an alternation of blotter-pressing paper-blotter, and so on. The press frames are on the top and bottom of the press, and it is then "locked up" by means of straps or stout cord.
  QUALITY CONTROL -- Select specimens that are free from evidence of insect feeding, rust infections, and other obvious pathological symptoms.
  QUALITY CONTROL -- Avoid depauperate individuals.
  QUALITY CONTROL -- Ensure that the specimen is either in flowering or fruiting condition.
  QUALITY CONTROL -- When an herbaceous specimen is collected, always include enough of the underground parts to show their character.

- Keeping wet material without its spoiling is a problem faced by collectors working in tropical regions, or under emergency situations when adequate drying facilities are lacking. Two techniques have been demonstrated as useful in these cases, but the results are inferior to those from the usual method of processing. In either case, the objective is to keep the material from decomposing after it has been collected and arranged in pressing papers, until such time as it can be dried by normal procedures. These two techniques are use of a solution of two parts of concentrated formaldehyde (40%) and three parts water, or use of a solution of one part of formaldehyde and two parts of 70 percent alcohol for temporary preservation of plant specimens before drying.

- Drying: There are two types of drying techniques: those accomplished without heat and those with the aid of artificial heat.
  QUALITY CONTROL -- No corrugate should be employed when using the drying technique without heat.
  QUALITY CONTROL -- Either technique can produce specimens of poor quality and because the drying process is much accelerated when heat is used greater care must be exercised during all its stages to produce quality specimens.

- Mounting: Usually specimens are mounted on sheets of standard size herbarium paper (11½ by 16½ inches). After mounting, they are stored in special cases built to fit sheets of this size. Herbarium papers in a selection of qualities are available from biological supply sources. Mounting is accomplished by the use of glue or paste, the use of adhesive linens, or the combination of both. There are three techniques most commonly used in mounting specimens with paste or glue. The first technique, the glass plate method, requires the use of a piece of plate glass at least 14 by 20 inches. The paste is spread thinly over most of the surface with a brush. The specimen is removed from the pressing sheet and placed face upward on the prepared plate, with all parts of the lower

side in contact with paste. It is then transferred carefully to the sheet of mounting paper. A pressing sheet of newsprint is placed over the specimen, pressed firmly, and taken off and discarded. Reapply fresh paste on the plate for each mounting. The second technique requires no glass plate. The paste is brushed directly on major protions of the specimen. The third technique is designed for mounting specimens with very light weight and thin texture. The specimen is laid, lower side uppermost, on a piece of cheesecloth, sprayed with a diluted solution of paste by means of an atomizer, and then flipped over onto the sheet of herbarium paper.
QUALITY CONTROL -- Use the longest-lasting and most durable paper for permanent museum collections.
QUALITY CONTROL -- Use special "A" Tin Paste and Improved Process Glue. Both products can be kept indefinitely when covered, and require no thinning or heating before use.
QUALITY CONTROL -- The glass plate should be kept clean for each mounting, and washed and set to dry after each mounting period.

- Labelling: For all specimens, whether pressed and mounted, preserved, or stored, herbarium labels are an essential part of its permanent preservation. The purpose of the label is to provide the user with pertinent information in relation to specimen.
  QUALITY CONTROL -- The label should be large enough to accomodate the data to be placed on it.
  QUALITY CONTROL -- Under no circumstances should a label be so large as to require folding.
  QUALITY CONTROL -- Data on labels should be typed. Data written on labels in longhand are always acceptable, but must be legible.

- Storing of fresh plant material for residual analysis of pesticides or other chemical substances.
  QUALITY CONTROL -- Use proper refrigeration equipment for storage.

Sample Preservation

- The preservation of herbarium collections from insect damages is accomplished most effectively by insecticides used in herbarium management including cyanide gas, paradichlorobenzene, carbon tetrachloride, or DDT. The two principal repellents used are naphthalene compounds and paradichlorobenzene.
  QUALITY CONTROL -- Use preservatives properly and cautiously.
  QUALITY CONTROL -- If liquid preservation is used, the plant material should be photographed in sufficient detail to show the form and such other significant details as may otherwise be lost.

- The preservation of juicy materials include the use of formaldehyde (5%), alcohol (70%), or aqueous hydroxyquinoline sulfate (1-2%).

- Quick-freezing techniques are also used for quantitative samples.

Sample Analysis

- Qualitative analysis: Identify the prepared plant specimens to the species level.
  QUALITY CONTROL -- Use available taxonomic references in relation to geographical flora.
  QUALITY CONTROL -- Use type specimens and consult experienced taxonomists for accurate identification.

- Productivity
  QUALITY CONTROL -- See Section 3.2.4.4 and 3.2.3.3.

3.2.4 Functional Tests

3.2.4.1 Culturing--

The objective of the culturing of organisms is to provide healthy organisms, i.e., disease-free and toxicant-free, for bioassays.

Assuming that organisms are transported under favorable conditions, stress-free, uncrowded and at favorable temperatures, from the field to the laboratory, these field-collected organisms must still be held in quarantine for at least seven days for observation for parasites and disease in order to avoid the transfer of such infections to the laboratory culturing tanks or living quarters. During this period, the organisms can recover from the stresses arising from treatment for disease or parasites during transit or upon arrival in the laboratory. Moreover, a sample of individuals can be used to determine if they have accumulated potential toxicants in their body tissue. This check becomes extremely necessary and crucial because toxicant-contaminated organisms, e.g., fish, are always more resistant if such toxicant is also used as a test substance.

During the quarantine period, the following quality assurance procedures must be carried out to ensure healthy organisms for bioassays:

- Organisms should be fed daily
- Crowding should be avoided
- Dead and abnormal organisms must be discarded. If the mortality is more than 10%, due to stress, parasites or diseases, destroy the lot. Clean and sterilize all affected containers and equipment, and collect another supply of organisms from a new area, if possible (Rand et al., 1975)
- Organisms should be observed carefully for unhealthy signs and closely attended by experienced personnel

Other important items that must be taken into consideration are:

- Laboratory animal management must be adequate. Adequate management, e.g., housing and care, permits animals to grow, mature, reproduce, or behave normally, and to be maintained in physical comfort and good health

- Personnel should be well trained and experienced and must care about the welfare of animals
- Animal facilities should be well designed and properly maintained. For example, the water supplies, freshwater or marine, are essential to assuring the success of rearing aquatic organisms

Culturing procedures and attendant quality control procedures for a number of organisms frequently used in bioassay follow.

- Phytoplankton, including freshwater and estuarine or marine algae

  References: Rand et al. (1975), pp. 697-703; U.S. EPA (1976b) pp. 19-25.

Quality Control:

o Proper adjustment of nutrient concentrations, pH, light intensity, and temperatures are essential prerequisites for the successful cultivation of algae.

o Sterilization must be done on the culturing utensils when preparing culturing media and whenever the algae are transferred.

o Use proper references that illustrate the instructions for the cultivation of the respective algae.

o Use available pure cultures from culture collections all over the nation or world. See Table 3.2.10.

- Protozoa, e.g., *Tetrahymena pyriformis*

  Reference: Rand et al. (1975), pp. 759-760.

Quality Control:

o Use standard bacteriological techniques to prepare and autoclave culture media and to transfer axenic cultures of *T. pyriformis*.

o Use available standard cultures. See Table 3.2.10.

o Maintain stock cultures at $26 \pm 0.5^{\circ}C$ in a suitable incubator, i.e., Revco Model IB-1650 from Revco, Inc., Scientific Industrial Division, 1100 Memorial Drive, West Columbia, S.C. 29169.

- Freshwater cladocerans, *Daphnia*

  References: Rand et al. (1975), pp. 763-764; Needham et al. (1937); Parker and Dewey (1969).

Quality Control:

o Use an appropriate culture medium for *Daphnia* culturing, e.g., manure-soil, a medium developed by Banta and modified by Anderson (1944).

o Once the cultures are initiated, the culture medium need not be changed.

o When the stock *Daphnia* reach old age and the reproductive rate drops, replace them with young females in fresh media.

- Marine copepod, Acartia tonsa

References: Rand et al. (1975), pp. 768-772; U.S. EPA (1976b); Heinle (1969); Mullin and Brooks (1967); Zillioux and Wilson (1966).

Quality Control:

o Use an appropriate diet and proper concentrations of diet for various stages of copepod.

o Use a generation cage that allows the eggs to pass through the net and hatch, eliminating the possibility of cannibalism by adults.

- Crustaceans, including grass shrimp, blue crabs, etc.

References: Rand et al. (1975), pp. 795-806; Hughes et al. (1974); Spotte (1970); Smith et al. (1974); Cook (1967); Mock (1974); U.S. EPA (1976b).

Quality Control:

o Use a favorable water supply and accomplish the control of competitors, predators and disease through filtration and sterilization by ultraviolet light treatment.

o Handle the test subjects carefully and as little as possible.

o Avoid cannibalism by holding young stages of crayfish in separate compartments.

o Routinely clean the sides and bottoms of compartments to remove organic material, growth, and wastes.

o Feed the newly hatched nauplii of brine shrimp, Artemia salina to the lobster larva to avoid cannibalism and to decrease the possibility of developmental variability.

o Control the essential environmental factors such as DO, temperature, and salinity as precisely as possible.

o The chelipeds of grass shrimps must be removed with fine surgical scissors to prevent removal of eggs by the females.

o The crustacean larvae should be removed from containers containing ovigerous females each morning and mixed together to insure uniformity of test animals.

- Larvae of aquatic insects, including those of stoneflies, mayflies, caddisflies, and Diptera.

References: Rand et al. (1975), pp. 829-830; Fremling (1967); Prater and Anderson (1976).

Quality Control:

o All insects collected must be examined for injuries before rearing in the laboratory.

o Avoid overfeeding which will cause DO difficulties.

o Supply suitable substrates for various insects, e.g., highly organic ooze for chironomids.

o Keep the water temperature under control for nymphal growth, e.g., the maintenance of temperature at 14° to 17°C for limited nymphal growth of mayflies.

o If dechlorinated, deionized tapwater is used, the water need never be drained and changed.

o To reduce the amount of turbidity, the charcoal filters should be flushed clean on a monthly basis and the charcoal replaced on a semi annual basis.

- Mollusks, such as oysters, clams, scallops and mussels

References(Rand et al. (1975), pp. 836-839; Loosanoff and Davis (1963); Castagna and Duggan (1971).

Quality Control:

o Provide an abundant water supply rich in planktonic food organisms.

o Clean regularly the intake pipe and the water system to insure that growth of fouling organisms in the pipes does not remove plankton organisms before the water reaches the holding tank.

o Clean accumulated feces and silt from the holding tray at least once a week, preferably twice a week.

o Thermal conditioning, e.g., induced spawning for scallops by raising the water temperature to 27° to 30°C, should be well controlled. Discard females once they have spawned.

- Fish

References: Rand et al. (1975), pp. 846-847, 849-853, 869-870; National Academy of Sciences (1973); Stalnaker and Gresswell (1974); Carlson and Hale (1972); Hokanson et al. (1973); McCormick et al. (1972); Siefert (1972); May (1970); Hirano and Oshima (1963); Hansen and Parrish (1977); Middaugh and Dean (1974); Middaugh and Lempesis (1976).

Quality Control:

o Limit the possibility of injuring fish during collection in the field. For example, the loss of some fish scales may cause disease problems raising fish mortality.

o Always avoid rearing fish in unusually high densities in the laboratory because disease bcomes a very important factor that can alter bioassay results or even nullify bioassays by killing the test subjects after they are weakened by the stress of the test substance or condition under study.

o Parasites and diseases must be controlled in order to get reliable bioassay results. Preventin of disease is preferred.

- Animals, including bids and mammals

References: "U.S. DHEW 1974"

Quality Control:

- Provide adequate veterinary care.
- Insure proper quarantine and isolation of animals.
- Be sure of absolute separation by species.
- Appropriate diagnosis, treatment, and control of diseases.

TABLE 3.2.9 MAJOR SOURCES OF STANDARD, PURE OR TYPE CULTURE COLLECTIONS FOR ALGAE AND PROTOZOA

Organisms	Source of Culture Collection
Algae	(1) Graduate School of Oceanography, University of Rhode Island, Narragansett, Rhode Island, U.S.A.
	(2) Department of Botany, University of Indiana, Bloomington, Indiana, U.S.A.
	(3) Eutrophication Research Program, Pacific Northwest Environmental Research Laboratory, 200 S.W. 35th Street, Corvallis, Oregon 97330, U.S.A.
	(4) Virginia Institute of Marine Science, Gloucester Point, Virginia 23062, U.S.A.
	(5) Chesapeake Biological Laboratory, Box 38, Solomons, Maryland 20688, U.S.A.
	(6) Dr. Robert Guillard, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, U.S.A.
	(7) The Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo, Japan
	(8) The Botany School of the University of Cambridge, Downing Street, Cambridge, Great Britian
	(9) Department of Botany, The Hebrew University of Jerusalem, Algal Laboratory, Jerusalem, Israel
	(10) Sammlung von Algenkulturen des Pflanzenphysiologischen Institute, Universitat Göttingen, Nikolansberger Weg 18, 34 Göttingen, Germany
	(11) Czechoslovak Academy of Sciences, Viniche 5, Praha 2, Czechoslovakia
Protozoa	(1) The American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.
	(2) The Botany School of the University of Cambridge, Downing Street, Cambridge, Great Britian
	(3) Czechoslovak Academy of Sciences, Vinicha 5, Praha 2, Czechoslovakia

3.2.4.2 Identification--

The published taxonomic works on organisms are comprehensive in scope and to list them here, even in condensed form for one specific organism, is not feasible. A reference list for the identification of the following aquatic organisms is given in "Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents" (U.S. EPA, 1973):

Organisms	Page No. of Section	
Phytoplankton	7, 8	PLANKTON
Zooplankton	12	PLANKTON
Periphyton	3	PERIPHYTON
Macrophyton	3	MACROPHYTON
Macroinvertebrates		MACROINVERTEBRATES
Coleoptera	33	
Diptera	34	
Crustacea	34	
Ephemeroptera	35	
Hemiptera	36	
Hirudinea	36	
Hydracarina	36	
Lepidoptera	36	
Megaloptera	36	
Mollusca	36	
Odonata	37	
Oligochaeta	37	
Plecoptera	37	
Trichoptera	37	
Marine macroinvertebrates		
Fish	16-18	FISH

In Section 3.2.7 is a bibliography which includes other organisms than those just mentioned and lists books, manuals or reports most frequently used in laboratories in the scientific community in which studies on organisms are in progress. This bibliography is organized in general taxonomic orders, i.e.:

Virus
Fungi
Bacteria and Actinomycetes
Protozoa
Other Microinvertebrates
Fish
Amphibia
Reptilia
Birds
Mammals
Plants
Aquatic Plants

The U.S. Environmental Protection Agency has prepared many identification manuals for selected organisms. For example, 11 identification manuals for aquatic macroinvertebrates have been prepared in the Agency's series, "Biota of Freshwater Ecology Systems" since the Agency's establishment (U.S. EPA

1976c). These are:

No. 1. Freshwater Planarians (Turbellaria) of North America
No. 2. The Genus Argulus (Crustacea: Branchiura) of the United States
No. 3. Freshwater Spaericean clams (Mollusca: Pelecypoda) of North America
No. 4. Freshwater Polychetes (Annelida) of North America
No. 5. Freshwater Amphipod Crustaceans (Gammaridea) of North America
No. 6. Aquatic Dryopoid Beetles (Coleoptera) of the United States
No. 7. Freshwater Isopoda (Asellidae) of North America
No. 8. Leeches (Annelida: Hirudinae) of North America
No. 9. Crayfishes (Astacidae) of North and Middle America, 1972
No. 10. Genera of Freshwater Nematodes (Nematode) of Eastern North America
No. 11. Freshwater Unionacean Clams (Mollusca = Pelecypoda) of North America.

They may be obtained without cost from the Aquatic Biology Section, Biological Methods Branch, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

According to EPA's Newsletter of Analytical Quality Control (April 1977, No. 33) the following identification manuals are being prepared by various taxonomic authorities:

- A key for the identification of 300^+ taxa of freshwater gastropods found in the North America (John B. Burch, Mollusk Division, Museum of Zoology, The University of Michigan, Ann Arbor, Michigan)

- A key to the identification of the common species of rotifers and a summary of their environmental requirements and pollution tolerance (John Gannon, University of Michigan Biological Station, Douglas Lake, Michigan)

- The classification, geographical distribution and ecology of the mussels of the United States (U.S. Environmental Protection Agency and Tennessee Valley Authority)

The availability of taxonomic references at the bench, and the skill and the systematic knowledge of the biologist, will determine the data quality resulting from identification efforts. No single biologist masters readily the sophisticated classification of living organisms, even an order of Insecta, e.g., Diptera. No single reference is completely appropriate for the Order Diptera. However, to ensure the validity and integrity of data in identifying organisms the biologist must be sure to do the following:

- Consult with appropriate experts for good, adequate bench references
- Use the available EPA identification manuals
- Develop and use a reference specimen collection (Weber, 1973)
- Utilize "outside" experts to solve difficult problems in specimen identification (Weber, 1973)
- Access the EPA "BIO-STORET" to verify the identification (Weber, 1976;

Nacht and Weber, 1976)

- Adopt computer data storage and retrieval system similar to "BIO-STORET" for geographical regions, e.g., Master Species for New England States

3.2.4.3 Counting--

The many and diverse schemes for counting numbers of organisms, that is, estimation of population size (numbers and density) fill a voluminous literature. THese methods have been briefly discussed and referenced by organism in Section 3.2.3.

The goal of population estimation appears to be twofold. First of all, one wishes to obtain the best possible estimates commensurate with the objectives of the study and the time, money, and personnel available. It is also desired to be able to make a statement about the precision of the estimate, i.e., how well the assumptions are met and the influence of sampling error. Overton (1971) gives considerable attention to the problems of collecting concomitant information to be used in validating assumptions, modifying the estimator if assumptions are ill-founded, and evaluating variances and confidence limits.

The quality control of counting includes the following activities:

- Apply a formal sampling plan. Count at least two samples. Use randomization in sample selection. Samples must be labelled with identification number and other related information when they arrive at the laboratory

- Train and organize personnel for quality. The same individual should be assigned to count the number of organisms throughout the study to optimize consistency of results

- Use the "total census" method whenever possible, to eliminate sampling errors. When other census methods are used, use them consistently throughout study period and compare the results from different methods. Each set of data should be accompanied by an explicit statement of constraints and definitions under which it is collected and by a critical evaluation of its "precision and accuracy"

- Utilize available automated counting equipment (counters) for counting of microorganisms

- Establish regular audits of performance in the field and laboratory

- Sign and witness all the data collected and all calculations

3.2.4.4 Biomass/Productivity--

Productivity and biomass should not be confused. Biomass is the summation of the weights of all individual organisms measured at a given time, while productivity is "rate of production". To avoid confusion, the time interval, e.g., year, month, etc., should be always stated when speaking of productivity.

The definition of productivity has been elaborated upon by Odum (1971) as follows:

- Primary productivity. The rate at which radiant energy is stored by photosynthethic and chemosynthetic activity of producer organisms (chiefly green plants) in the form of organic substances which can be used as food materials.

- Gross primary productivity. The total rate of photosynthesis, including the organic matter used up in respiration during the measurement period. This is also known as "total photosynthesis" or "total assimilation".

- Net primary productivity. The rate of storage of organic matter in plant tissues in excess of the respiratory utilization by the plants during the period of measurement. This is also called "apparent photosynthesis" or "net assimilation".

- Net community productivity. The rate of storage of organic matter not used by heterotrophs (net primary production - heterotrophic consumption) during the growing season.

- Secondary productivities. The rate of energy storage at consumption levels.

Methods for measuring productivity are summarized in Table 3.2.10.

3.2.4.5. Physical Characteristics of the Environment--

The principal physical characteristics of the environment that are of interest are temperature, color, turbidity (or suspended solids), oil and grease and airborne particulates. Water temperature is among the more important of these characteristics because:

- The water covers a major part of the earth. The life associated with the water environment has its species composition and activity regulated by water temperature. Essentially all of the organisms are "cold-blooded" or poikilotherms. The temperature of the water regulates their metabolism and their ability to survive and reproduce effectively.

- Industrial uses by man for process water and for cooling are likewise regulated by the temperature of the water. According to a report by the Federal Water Pollution Control Administration (1967), "Temperature, a catalyst, a depressant, an activator, a restrictor, a stimulator, a killer, is one of the most important and most influential water quality characteristics to life in water."

Standard experimental protocols for testing physical characteristics in the field do not appear to have been developed: Nakatani (1969) believes that "the best practical method to investigate the effects of elevated temperatures on salmon or other desirable species in the Columbia River is

TABLE 3.2.10 METHODS FOR MEASURING PRODUCTIVITY

Methods	Uses	Quality Control
Harvest method	Terrestrial plants such as: Cultivated crops, e.g., wheat, corn, rice. Noncultivated ragweed field, or where plants are little consumed until growth has been completed. Young forests or crop-like forest plantations. Cattle range (expressed in terms of the number of cattle that can be supported by so many acres).	This method can only be used in situations in which herbivore animals are not important and in which a steady-state condition is never reached. Since food used by the plants themselves and associated microorganisms and animals is not included, this method always measures net community production.
Oxygen measurement e.g., "dark-and-light" bottle method, diurnal curve method	Phytoplankton, macrophyton in freshwater ecosystems and in marine ecosystems.	"Dark-and-light" bottle method must be limited to a short duration, e.g., one 24-hour cycle or less. The combination dark-and-light bottles measure primary production, and the light bottle measures net community production. The use of large plastic spheres instead of bottles reduces the inner surface-to-volume ratio and is presumed to reduce the effect of surface bacterial growth. The "diurnal curve" method is particularly applicable to streams or estuaries and is especially useful in dealing with polluted waters. It measures gross primary production. Reasonable corrections should be made for a source of errors introduced by diffusion, if any.
Carbon dioxide method e.g., enclosure method	Terrestrial plant communities, such as crops, grasslands, etc.	Equivalent to the aquatic "dark-and-light" bottle method, enclosure method measures gross and net primary production. Refrigerating or air

(continued)

TABLE 3.1.10 (Continued)

Methods	Uses	Quality Control
		conditioning the chamber often becomes necessary if measurements are to extend over an appreciable period of time. As in the diurnal curve method, the accuracy of the aerodynamic method depends on the accuracy of the corrections that must be made for mass movements of air and for gas evolution from soil that may contain CO_2, which is not a product of metabolism during the period of measurement. Use of remote sensing and continuous monitoring techniques should increase the validity and integrity of data.
Radioactive methods (^{14}C, ^{32}P are used)	Aquatic plants, phytoplankton.	The ^{14}C method is one of the most sensitive and widely used methods for measuring aquatic plant production (radioactive carbon [^{14}C] added as carbonate). Use precisely and adequately calibrated radioactive counting device. Trained, experienced personnel should be assigned on the control and handling of radioactive material
pH method	Aquatic ecosystems, laboratory micro-ecosystems.	The investigator must first prepare a calibration curve for the water in the particular system to be studied because (1) pH and CO_2 content are not linearly related, and (2) the degree of pH change per unit of CO_2 change depends on the buffering capacity of the water. See Beyers, et al., 1963, Publ. Inst. Mar. Sci. Univ. Texas 9:454-489, and Beyers, 1964, Amer. Bio. Teacher 26:491-498 for the details of a pH calibration curve. Use precise instrumentation of remote sensing and continuous monitoring techniques.

(continued)

TABLE 3.2.10 (Continued)

Methods	Uses	Quality Control
Disappearance of raw materials such as phosphorus and nitrogen	Marine phytoplankton.	This method measures the net productivity of the whole community during the period of spring growth of phytoplankton. The method must be used with caution since non-living forces may also cause the disappearance of these raw materials.
Chlorophyll method	Aquatic communities, such as phytoplankton, macrophyton, and terrestrial communities.	This method measures primary productivity. Follow standard extracting (pigment) procedures. Spectrophotometer must be regularly and adequately calibrated for precision.

TABLE 3.2.11 PHYSICAL CRITERIA FOR WATER QUALITY
(NAS, 1974; U.S. EPA, 1976d)

SOLIDS (SUSPENDED, SETTLEABLE) AND TURBIDITY

Freshwater fish and other aquatic life: Settleable and suspended solids should not reduce the depth of the compensation point for photosynthetic activity by more than 10 percent from the seasonally established norm for aquatic life.

COLOR

Waters shall be virtually free from substances producing objectionable color for aesthetic purposes.

The source of supply should not exceed 75 color units on the platinum-cobalt scale for domestic water supplies.

Increased color (in combination with turbidity) should not reduce the depth of the compensation point for photosynthetic activity by more than 10 percent from the seasonally established norm for aquatic life.

OIL AND GREASE

For domestic water supply: Virtually free from oil and grease, particularly from the tastes and odors that emanate from petroleum products.

For aquatic life:
(1) 0.10 of the lowest continuous flow 96-hour LC50 to several important freshwater and marine species, each having a demonstrated high susceptibility to oils and petrochemicals.
(2) Levels of oils or petrochemicals in the sediment which cause deleterious effects to the biota should not be allowed.

(3) Surface waters shall be virtually free from floating nonpetroleum oils of vegetable or animal origin, as well as petroleum-derived oils.

TEMPERATURE

Freshwater aquatic life: For any time of year, there are two upper limiting temperatures for a location (based on the important sensitive species found there at that time).
(1) One limit consists of a maximum temperature for short exposures that is time dependent and is given by the species-specific equation:

$$T = (1/b) \ \log_{10} (t-a) - 2°C$$

where T = temperature (°C)
b = slope of the line fitted to experimental data and available from Appendix II-C, NAS, 1974, for some species
$\log_{10}$ = logarithm to base 10 (common logarithm)

(continued)

TABLE 3.2.11 (Continued)

t = time (minutes)
a = intercept on the "y" or logarithmic axis of this line fitted to experimental data and which is available from Appendix II-C, NAS, 1974, for some species

(2) The second value is a limit on the weekly average temperature that:
a. in the cooler months (mid-October to mid-April in the north, and December to February in the south) will protect against mortality of important species if the elevated plume temperature is suddenly dropped to the ambient temperature, with the limit being the acclimation temperature minus 2°C when the lower lethal threshold temperature equals the ambient water temperature (in some regions this limitation may also be applicable in summer);
b. in the warmer months (April through October in the north, and March through November in the south) is determined by adding to the physiological optimum temperature (usually for growth) a factor calculated as one third of the difference between the ultimate upper incipient lethal temperature and the optimum temperature for the most sensitive important species (and appropriate life state) that normally is found at that location and time; or
c. during reproductive seasons (generally April through June and September through October in the north, and March through May and October through November in the south) the limit is that temperature that meets site-specific requirements for successful migration, spawning, egg incubation, fry rearing, and other reproductive functions of important species. These local requirements should supersede all other requirements when they are applicable.
d. There is a site-specific limit that is found necessary to preserve normal species diversity or prevent appearance of nuisance organisms.

Marine aquatic life: In order to assure protection of the characteristic indigenous marine community of a water body segment from adverse thermal effects, the following must be observed:

a. the maximum acceptable increase in the weekly average temperature due to artificial sources is 1°C (1.8°F) during all seasons of the year, providing the summer maxima are not exceeded; and
b. daily temperature cycles characteristic of the water body segment should not be altered in either amplitude or frequency.

Summer thermal maxima, which define the upper thermal limits for the communities of the discharge area, should be established on a site-specific basis. Existing studies suggest the following regional limits:

	Short-term Maximum	Maximum True Daily Mean*
Sub-tropical regions (south of Cape Canaveral and Tampa Bay, Florida, and Hawaii)	32.2°C (90°F)	29.4°C (85°F)

(continued)

TABLE 3.2.11 (Continued)

	Short-term Maximum	Maximum True Daily Mean*
Cape Hatteras, N.C., to Cape Canaveral, Florida	32.2°C (90°F)	29.4°C (85°F)
Long Island (south shore) to Cape Hatteras, N.C.	30.6°C (87°F)	27.8°C (82°F)

* True Daily Mean = average of 24 hourly temperature readings

Baseline thermal conditions should be measured at a site where there is no unnatural thermal addition from any source, which is in reasonable proximity to the thermal discharge (within 5 miles) and which has similar hydrography to that of the receiving waters at the discharge.

the direct approach of working on-site, using local fish and Columbia River water". So he drifted juvenile chinook salmon (0-age) in live-box through the plumes produced by the Hanford Reactor in the Columbia River and warmed shoreline areas, used an inclined plant scoop-trap in the river downstream from a reactor outfall to sample the natural run of seaward migrants, and scored mortalities. In both the live-box drifts and the trap collections, no mortalities attributable to heat were observed. The water temperature observed at the fish trap anchored about 400 meters downstream in a center of a reactor discharge plume showed a range of 10.5^{o} to 15.5^{o}C.

In addition to cage or trap studies, biologists have suggested other means to study the effect of heated effluents on anadromous fish by counting natural fish populations or observing fish swimming behavior (or runs). Observations are made using aerial surveys by planes or using sonic tags on fish (Nakatani, 1969), and direct observations on fish in a runway or channel (Alabaster, 1969).

Table 3.2.11 lists the physical criteria for water quality (U.S. EPA, 1976d; National Academy of Sciences, 1974).

3.2.5 Field Bioassay

3.2.5.1 Aquatic Field Tests--

Three terms are often used to describe field tests. These are "field survey", "monitoring program" and "field test" (Livingston et al., 1974; U.S. EPA, 1975a).

- "In a field survey, a sampling method is devised to include: a broad range of the animal and plant life, both perturbed and unperturbed areas, seasonal changes; and where possible, before and after effects of some event, such as the application of a potentially registrable pesticide."

- Monitoring implies continuous measurement of some variable.

- "In field tests, organisms are maintained in cages or confined areas in the field environment. Such systems can continually assess the effect of the application on a series of representative species."

A portion of the real world can be partitioned off and purposely contaminated. The advantages of this type of research are that the spill or contamination is under control of the investigator so that pre-stress data can be assembled and the actual stress manipulated and measured, while the complexity of the real world is retained to a greater degree than in a laboratory study. The problems are:

- Deciding whether deliberate damage to even a small portion of environment is justified by the information that will be obtained;
- Confining the damage to the area under study;
- Deciding whether the portion of the environment under study is representative; and
- Achieving sufficient control over the test area (U.S. EPA, 1975a).

However, it appears that the approach with the greatest possibility for standardization is cage-type or confined-area exposure.

EXAMPLE: AQUATIC FIELD TESTING

Design of Experiment

- The design of the experiment is one of the first tasks in aquatic field testing and a crucial factor to achieving the ultimate goal -- "Quality Assurance" in the field test.
- Use statistical consultation in the design of the experiment. At the very least, suitable replication and control areas are a must, and the value of pre-application field data becomes obvious.
- Apply a formal sampling plan. Notice the great difficulties of sampling with mobile species and species with nonrandom distribution. Different communities and localities may require different sampling procedures. The frequency of sampling depends greatly on the objective of the study. For example, the "reproductive success" study of an individual species requires less frequent sampling than the mortality study of the same species.
- Choose an appropriate test area. The area to be used should be as homogeneous as possible with respect to the biotic, physical and chemical environment. Every effort should be made to choose an area which allows the investigator to prevent, control, or minimize the spread of the applied toxicant.
- A high degree of knowledge of the biology of the various species is required.

Personnel

- The team which conducts the field test must be adequately organized. It should consist of at least one aquatic toxicologist or biologist as team supervisor, one or two technicians, and have access to an analytical chemist and a biostatistician.

- Train all personnel for quality.

Facilities and Equipment

- The maintenance of field equipment and/or instruments and cage construction are major concerns. Among these the cage construction is of paramount importance for the field toxicity test.

- Cage construction: The construction of cages for specific taxa depends on the species, its predators, the habitat, and the properties of the chemical being tested. Heitmuller and Nimmo (1972) constructed a holding cage for exposing penaeid shrimp to bottom sediment and suggested that the cage is also suitable to hold mollusks, crabs, or fish for field tests.

- Bioassay trailer: A bioassay trailer (Zillich, 1969) has been proven useful for testing the biological effects of many industrial wastes in the field. Federal, State and local agencies have just begun utilizing mobile bioassay units in applied research areas as aids for engineering design, in investigations to determine water quality criteria, in enforcement of water quality standards, and in aquatic pest control studies. Ideally, the design of these units should be guided first by the mission of the sponsor and then by considerations of economy and flexibility (Gerhold, 1973).

- Portable apparatus for acute toxicity bioassays: The apparatus is simply designed for conducting acute toxicity bioassays in the field, particularly effluent tests. Falk (1973) designed an apparatus which proved to be very satisfactory under field conditions, being inexpensive, light, and portable as well as giving satisfactory results.. Fish in the control tests survived with no mortality. Through mixing by aeration, the temperature did not vary more than 2°C over a 96-hour period. Results obtained from experiments were comparable with those obtained from bioassays conducted under controlled laboratory conditions. Additional advantages of this portable apparatus were: it is much cheaper to set up than the controlled laboratory for the effluent. Burress (1975) employed large plastic bags to contain 284 liters of water and used more and larger fish in 96-hour tests of antimycin without employing either aeration devices or bulky supports for rigid vessels as indicated in Falk (1973). Burress highly recommended his method for conducting on-site toxicity tests.

Test Methods

- According to the Federal Register (Vol. 40, No. 123, June 25, 1975), "no universally applicable methods are available for field testing of pesticides because of the wide diversity of mechanisms whereby a pesticide may enter the environment, the diversity of habitats which may be affected and the nature of the pesticide (solubility in water, degradability, etc.)." The U.S. Environmental Protection Agency has indicated field methods as "developmental". That means the method has been proposed by one or a few toxicologists and has been used to test only a few compounds by the original researcher. "There is no concensus that the method provides appropriate data or that modifications of the method would not be appropriate. Techniques involved may not be well known to other toxicologists, and therefore other toxicologists may require considerable experience with the method before they can obtain consistent results."

- Other than the complexity of the environment and the unique nature of each chemical, the inherent difficulties of sampling and biological variability encountered in the field have hindered the progress of field chemical tests.

- In spite of major difficulties, it is expected that as the theoretical and practical aspects of environmental research improve, there will be a capability to measure the effects of single and combined factors under field conditions. The approaches to become "routine" methods are most likely applicable to cage-type (C) exposure or confined-area (CA) exposure. See Table 3.2.12

Test Subjects

- Some general considerations in the selection of test subjects are (U.S. EPA, 1975a):

  - o Be realistic in choice of species. Species collected locally will normally be easier to work with.

  - o Be aware of the possibility of induced resistance.

  - o Should caged animals be used, an adequate period of acclimation is necessary.

  - o Within the constraints of acceptable techniques, choose the most sensitive species and/or life stage inhabiting that ecosystem.

  - o The species must be readily available.

  - o Whether organisms are collected directly or purchased, every effort should be made to insure that they are healthy and are not subjected to unnecessary stress. See Perkins (1972) on discussion of the importance of stresses such as collecting, handling and maintenance.

  - o Collection techniques described in "Biological Field and Laboratory Methods," U.S. EPA (1973), should be used.

- From Table 3.2.12, the most common species of fish used in cage-type field testing of toxic pollutants from the marine environment are as follows: sheepshead minnow (*Cyproinondon variegatus*), striped mullet (*Mugil cephalus*), mosquitofish (*Gambusia affinis*), sailfin molly (*Mollienesia latiphinna*) and killifish (*Fundulus heteroclitus*). From the freshwater environment the representative ones are: Largemouth bass (*Micropterus salmoides*), bluegill

(*Lepomis macrochirus*), fathead minnow (*Pimephales promelas*), rainbow trout (*Salmo gairdneri*), and brook trout (*Salvelinus fontinalis*).

- A similar list of invertebrates would include:

Marine - Blue crab (*Callinectes sapidus*)
Fiddler crab (*Uca minax*, *U. pufnax*)
Grass shrimp (*Palaemonetes pugio*)
Eastern oyster (*Crassostrea virginica*)

Freshwater - The limited studies (cage-type) suggest no common species.

TABLE 3.2.12 AQUATIC SPECIES OR TAXA, FRESHWATER AND MARINE, USED IN FIELD CAGES (C) OR CONFINED-AREA (CA) TYPE STUDIES

Species	Type of Study (C or CA)	References
FRESHWATER FISH:		
Brown trout (*Salmo trutta*)	C	Adams, 1975
Bluegill (*Lepomis macrochirus*)	CA	Andrews et al., 1966
Black crappie (*Pomoxis vigro-maculatis*)	C	Bridges, 1958
Bluegill		
Black bullhead (Ictalurus melas)		
Carp (*Cyprinus carpio*)		
Flier sunfish (*Centrachus macropterus*)		
Golden shiner (*Notemigonus crysoleucas*)		
Goldfish (*Carassius auratus*)		
Green sunfish (*Lepomis cyanellus*)		
Grass pickerel (*Esox americanus*)		
Largemouth bass (*Micropterus salmoides*)		
Longnose gar (*Lepisosteus osseus*)		
Mosquitofish (*Gambusia affinis*)		
Smallmouth buffalo (*Ictiobus bubalus*)		
Steel-colored minnow (*Notropis whippli*)		
Swamp darter (*Ethoestoma gracile*)		
Warmouth (*Chaenobryltus gulosus*)		
White crappie (*Pomoxis annularis*)		
Yellow bullhead (*Ictalurus netalis*)		
Minnows	C	Carpenter, 1925
Trout		
Brown trout	CA	Dacre and Scott, 1973

(continued)

TABLE 3.2.12 (Continued)

Species	Type of Study (C of CA)	References
Largemouth bass	C	Eipper, 1959
Bluegill		
Golden shiner		
Fathead minnow (*Pimephales promelas*)		
Rainbow trout (*Salmo gairdneri*)		
Brook trout (*Salvelinus fontinalis*)		
Java fish (*Puntinus javanicus*)	C	Gorbach et al., 1971
Bluegill	CA	Hemphill, 1954
Bonytail (*Gila robusta elegans*)		
Bullhead		
Brown trout		
Carp		
Largemouth bass		
Black bullhead	CA/C	Kallman et al., 1962
Channel catfish (*Ictalurus punctatus*)		
Greek chub (*Semotilus atromaculatus*)		
Green sunfish		
Fathead minnow		
Rainbow trout		
Smallmouth bass		
White sucker (*Catostomus commersoni*)		
Yellow bullhead		
Bluegill	CA	Lawrence, 1950
Goldfish		
Largemouth bass fingerling		
Bigmouth buffalo (Ictiobus *cyprinellus*)	CA	Mayhew, 1959
Bluegill		
Black bullhead		
Black crappie		
Carp		
Channel catfish		
Gizzard shad (*Dorosoma cepedianum*)		
Largemouth bass		
Guillback (*Carpiodes cyprinus*)		
Yellow bass (*Roccus mississippiensis*)		
Snakeskin gourami (*Trichogaster pectoralis*)	C	Moulton, 1974
Mosquitofish	CA	Mulla, 1962b

(continued)

TABLE 3.2.12 (Continued)

Species	Type of Study (C of CA)	References
Atlantic salmon	CA	Sprague et al., 1965
Green sunfish	CA	Summerfelt and Lewis, 1967
Kwi Kwi (*Haplosternum littorale*) Srieba (*Astyanax bimaculatus*) Krobia (*Cichlasoma bimaculatum*)	CA	Vermeer et al., 1974
Utah chub (*Gila atravia*)	CA	Workman and Newhold, 1963
Leatherside chub (*Synderichthys* sp.) Dace (*Rhinichtys* sp.)		
FRESHWATER INVERTEBRATES:		
Gastropod Diptera Odonata Ephemeroptera Coleoptera Hemiptera	CA	Andrews et al., 1966
Copepod Cladocera Rotifera	CA	Coswell, 1965; Eipper, 1959
Louisiana red crawfish	C	Hendrick and Everett, 1965
Protozoa Rotifera Entomostraca	C	Hoffman and Olive, 1961
Aquatic insects Water mites Midges, etc.	CA	May et al., 1973
Mayflies (Ephemeroptera) Caddisflies (Trichoptera) Elmid beetles (Elmidae) Midges (Chironomidae)	C/CA	Moye and Luckmann, 1964

(continued)

TABLE 3.2.12 (Continued)

Species	Type of Study (C or CA)	References
Texas Snails (*Tropicorbis sp.*)	CA	Nolan and Berry, 1949
Plankton, Benthic Invertebrates	CA	Tarzwell, 1948
Egyptian snails: *Balinus trunccatus* *Biomphalaria alexandrina* *Physa* sp.	C	Unrau et al., 1965
South American Snail (*Pomacea* sp.)	CA	Vermeer et al., 1974
OTHER FRESHWATER ORGANISMS		
Frog and Toad	CA	Mulla, 1962a
Bullfrog - Tadpoles (*Rana catesbeiana*)	CA	Mulla, 1962b
Plants: *Lemna* *Alisma* *Sagittaria* *Chara* *Potamogeton* Algae		Eipper, 1959
Frog (*Pseudis paragoxa*)	CA	Vermeer et al., 1974
MARINE FISH		
Diamond killifish (Adenia xenica)	CA	Croker and Wilson, 1965
Darter goby (*Gobionellus bolcosoma*)		
Gulf killifish (*Fundulus grandius*)		
Killifish (*Fundulus* sp.)		
Longnose killifish (*Fundulus similis*)		
Mosquitofish (*Gambusia affinis*)		
Rainwater killifish (*Lucania parva*)		
Sailfin molly (*Mollinesia latipinna*)		
Sheepshead minnow (*Cyprinodon variegatus*)		
Spot (*Leiostomus xanthurus*)		
Striped mullet (*Mugil cephalus*)		
Tidewater silverside (*Menidia beryllina*)		
Mummichog (*Fundulus heteroclitus*)	C	George et al., 1957
"Variegated cyprinodon" (*Cyprinodon variegatus*)		
Spot		
White mullet (*M. curema*)		

(continued)

TABLE 3.2.12 (Continued)

Species	Type of Study (C or CA)	Reference
Mullet (M. Cephalus) = Striped mullet	C	Ludwig et al., 1968
Croakers (Leiostomus xanthurus)		
Broad killifish (C. variegatus)		
Gulf killifish		
Cyprinodon sp.	C/CA	Springer and Webster, 1951
Fundulus sp.		
Sheepshead minnow	C	Tagatz et al., 1974
Flounder (Paralichthys sp.)	C	U.S.D.I., 1967
Mullet (M. cephalus)		
Puffer (Sphaeroides sp.)		
Sailfin molly		
Pinfish	C	U.S.D.I., 1968
Sheepshead		
Drum		
Mollies		
Fundulus		
MARINE INVERTEBRATES		
Fiddler crab (Uca minax)	C	Croker and Wilson, 1965
Blue crab (Callinectes sapidus)		
Marsh fiddler (Uca pugnax)		George et al., 1957
Red-jointed fiddler (U. minax)		
Marsh crab (Sesarma reticulatum)		
Blue crab (C. sapidus)	CA	Koenig et al., 1976
Marine mussel (Mytilus edulis)	C	Lee et al., 1972
Blue crab	C	Ludwig et al., 1968
Soft shell clam (Mya arenaria)	C	Rawls, 1965
Blue crab		
Eastern oyster (Crassostrea virginica)		
Blue crab	CA	Springer and Webster, 1951
Marsh fiddler	C	Springer and Webster, 1951
"Bait" shrimp (Palaemonetes pugio)		
Blue crab		

(continued)

TABLE 3.2.12 (continued)

Species	Type of Study (C or CA)	References
Grass shrimp (Palaemonetes vulgaris) Pink shrimp (P. pugio) Snail (Littorina irrorata)	C	Tagatz et al., 1974
White shrimp (Penaeus setiferus) Blue crab Fiddler crab (Uca sp.) Oysters (Crassostrea sp.)	C	U.S.D.I., 1967
"Bait" shrimp (P. pugio) Fiddler crab Blue crab	C	U.S.D.I., 1968

3.2.5.2. Non-aquatic Field Tests--

Dr. J. L. Lincer of the Mote Marine Laboratory has compiled some protocols for wildlife toxicology and hazard evaluation for the Environmental Protection Agency (U.S. EPA, 1975b). These protocols include:

- Protocol for determination of the approximate maximum tolerated dose
- Protocol for laboratory acute oral toxicity - Birds
- Protocol for determining lethal dietary concentrations of chemicals to birds (5-day dietary LC50)
- Protocol for evaluation of reproductive effects of pesticides on the mallard
- Protocol for laboratory acute dermal toxicity test
- Protocol for small pen simulated field test to evaluate pesticide hazards to birds
- Protocol for large pen simulated field studies
- Protocol for full-scale field tests to evaluate pesticide hazards to wildlife.

As toxicity tests move from the laboratory to full-scale field tests, it becomes more necessary, but more difficult to control important variables. Simulated field tests, both small-pen and large-pen, furnish intermediate data to evaluate wildlife toxicity under semi-natural conditions. Simulated field tests should follow acute and subacute toxicity studies. Large-pen simulated field tests have been used to measure chronic effects, including those on reproduction. Data from laboratory toxicity tests and simulated field tests are serviceable in designing a full-scale (or unrestricted) field test. The unrestricted field test must necessarily follow both acute and subacute toxicity tests and simulated field tests. This test produces data on actual commercially treated pesticide target areas where non-target wildlife

live unshackled in their feeding, reproductive and other activities (U.S. EPA, 1975b)

Examples of protocols for Small Pen Simulated Field Test, Large Pen Simulated Field Studies, and Full-scale Field Tests to Evaluate Pesticide Hazards to Wildlife are given in the following pages.

EXAMPLE: SMALL PEN SIMULATED FIELD TEST

Purpose of Study

- Avian toxicity -- To evaluate pesticide hazards to birds.

Materials

- Bobwhite (this protocol has been developed as an initial simulated field test for this bird). With modifications, other species could be tested.
- The test subjects shall be obtained from pen-reared stock.

Design of Experiment

QUALITY CONTROL -- Use statistical consultation in the design of the experiment.
QUALITY CONTROL -- Use good supervisory practices to ensure that protocols are followed.

- Quarantine period. All birds shall be maintained in outdoor pens, in the general area where the field test is to be conducted, for at least 2 weeks prior to the test.
- Number of birds. Each test should contain not less than six pairs of birds per control group and not less than six pairs of birds per test group, with one pair of birds per pen.
  QUALITY CONTROL -- It is recommended that at least 12 additional birds be procured and held in outdoor pens for replacement purposes.
- Pens (size, construction, etc.). Each pen shall contain approximately 1.8 m^2 (20 ft^2). Suitable pen dimensions might be 1.20 m by 1.50 m (4 ft by 5 ft) or 0.90 m by 2.10 m (3 ft by 7 ft) and 0.30 m (1 ft) high. The pens should consist of a wooden frame made from 4 cm by 4 cm (2 in by 2 in) lumber and covered on the inside with 1 cm (½ in) mesh hardware cloth. Pen height may be increased to a height that will accomodate vegetation growth through the test period. Pens should have an opening through which birds can be removed or added. Each pen should contain a poultry waterer, preferably a 1-liter (1-quart) chick fount and a small box, 30 cm by 30 cm by 25 cm (12 in by 12 in by 8 in), open on one side, to serve as a shelter for the birds.
  QUALITY CONTROL -- To avoid possible contamination, scrub wire and replace frames if pens have been used for previous testing. Use of aluminum tubing for framing will make cleaning of pens easier.

QUALITY CONTROL -- Do not cover pen bottom. Stake pen securely to the ground to minimize predation.
QUALITY CONTROL -- Movepens daily, or as required to maintain adequate vegetation cover.

- Test Conditions. Conditions for evaluation of each pesticidal formulation should approximate those to be encountered in the routine use of the product. For example, evaluation of a cotton insecticide should be made in a cotton field, and the timing, rate, number, and manner of applications should be identical with those for control of cotton insects.
  QUALITY CONTROL -- Handle (feeding, watering and observation) the control birds the same as the test birds.
  QUALITY CONTROL -- Clearly mark all pens and all birds to assist accuracy in data collection.
  QUALITY CONTROL -- Care should be taken at all times to avoid possible contamination through drift from adjacent areas or from improper cleaning of equipment.

Conduct of Experiment

- Place pens and shelters in positions, and introduce birds (1 male and 1 female per pen) prior to application of pesticide.
  QUALITY CONTROL -- Establish regular audits of performance.
  QUALITY CONTROL -- Sufficient food and water are to be available to the birds at all times, other than during the indicated 12-hour period.

- Place filled waterers and about 100 g (3 to 4 ounces) of cracked corn, wheat or other grain in 1/3 of the total test and control pens used prior to test. The remaining test and control pens are to be left without feed and water for 12 hours after the pesticide application, at which time feed and water are to be introduced to these pens, as above.
  QUALITY CONTROL -- Follow all safety precautions, as specified on the product label, when entering the treated field.

- Observation of Test: If either member of the pair dies, the survivor is to be removed, placed in an individual holding pen, and a fresh pair placed in the pen. The survivor should be observed until death or for 14 days. Sacrifice survivors, including "control" group and birds held for replacement at the termination of the experiment.
  QUALITY CONTROL -- Same individual should be assigned on the routine observation job, and at the same time period each day the observations should be made.

- Duration of test: For pesticides which are to be applied once per season, tests are to be continued for not less than 14 days. For pesticides which are to be applied more than once per season, tests are to be continued for 14 days after the final application, with movement of pens immediately prior to each application.

Reporting of Data

- Appropriate items to be considered are as follows:
  - Location of test
  - Dates
  - Weather data
  - Species, sources, age, medical and chemical administration history, body weight, weight changes of birds; individual identification
  - Chemical formulation, rate of application, manner of application
  - Vegetative cover, residue analysis
  - Pen description, pen placement
  - Diet, food and water supply schedule, feed consumption
  - Visual signs of intoxication, accidental deaths, or injuries
  - Replacement schedule
  - Gross pathological or histological examinations
  - Statistical methods.

  QUALITY CONTROL -- Use statistical expertise in analysis of results.
  QUALITY CONTROL -- Adopt a system for review and publication of data and reports.

Reference

- U.S. EPA, 1975. Guidelines for Registering Pesticides in the United States, Appendix, Part VII - Hazard Evaluation, Subpart C: Wildlife Toxicology. Federal Register, Vol. 40, No. 123 - Wednesday, June 25, 1975, pp. 26920-26921.

EXAMPLE: LARGE PEN SIMULATED FIELD STUDIES

Purpose of Study

- Avian Toxicity - To determine pesticide effects on birds under semi-natural conditions and to assess the degree of hazard presented by the formulation and application rates of pesticides being considered for registration.

Materials

- Bobwhites, ring-necked pheasants or other species.

Design of Experiment

QUALITY CONTROL -- Use statistical consultation in design of experiment.

- Size of pens: wire-covered pens should be constructed covering a minimum ground area of $45 m^2$ ($500 ft^2$) per pen. Suitable pen dimensions might be 3 m or 3.5 m by 15 m or 23 m (10 ft or 12 ft by 50 ft or 75 ft), with the top cover at a height of about 2.0 m (6.5 ft). Other dimensions covering

$45m^2$ ($500 ft^2$) or more per pen may be used.
QUALITY CONTROL -- Before pens are planned and constructed, the designer and builder should consult wildlife agencies and successful game farms to learn practical consideration such as prevention of disease and parasites, soil drainage requirements, support of top cover to prevent collapse under the weight of snow, types of watering, etc.

- Number of cages: 24 to 36 pens are sufficient to test one chemical. This would provide 6 to 9 control pens and 6 to 9 pens for each of 3 treatment levels (the proposed treatment rate and 2 multiples of that rate such as 3 or 5X and 5 or 10X). An independent water supply and a small shelter should be furnished in each pen. Metal flashing should be placed around all pens to a height of about 45 cm (18 in) above ground and to a depth of about 15 cm (6 in) below the ground surface.
  QUALITY CONTROL -- Double the size of pens used when pheasants are utilized in this experiment. For example, two 3.7-by 22.9-m pens could be converted to one 3.7-by 45.7-m pen.

Birds

- If bobwhites are used, pens may be stocked with 1 mated pair per pen. One-year-old birds of known history, not previously exposed to pesticides, shall be placed in the pen at least 2 weeks prior to the pesticide applications. If pheasants are used a pen should be stocked with 1 male and 5 females per larger pen.
  QUALITY CONTROL -- A supply of replacement birds should be maintained in outdoor pens near the control pens.
  QUALITY CONTROL -- All birds must be in healthy condition prior to the test.

Test Conditions

- Pen position: Keep pens under conditions as natural as possible. Use movable pens that can be set up over the crop or vegetation on which the pesticide will be applied. If nonportable pens are used, then soil should be suitable for growing the pertinent crop or vegetation.
  QUALITY CONTROL -- For statistical purposes, randomize the test pens. Before randomization, stratify the treatment pen locations first because of the drift problem of pesticides.

- Pesticide administration: Handspray the pesticide at the same rate, timing, number of applications, and formulation as outlined in the proposed registration. Replicate pens should also be treated at two multiples (such as 3 or 5X and 5 or 10X) of the rate requested in the petition.
  QUALITY CONTROL -- Spraying should be done under minimum wind conditions and with protective shielding to prevent contamination of adjacent sprayed pens and/or control pens.

- Feed and water: These should also be treated at the 3 rates. The food treatment rates can be based on results of residue studies required for other purposes in the registration procedure. Treated food should be prepared within 1 day of the time environments are sprayed. Treated food should be supplied daily or every other day to the test birds in feeders protected from the weather.

  Another desirable phase of the test would be to provide treated animal foods such as grasshoppers or other invertebrates (earthworms, etc.) to the penned birds simultaneously with the pen environment application.

  Various combinations of treatments can be made to determine the major route of pesticide exposure to the test birds as follows:

  (1) Pen environment only with "clean" food and water.
  (2) "Clean" pen environment and water and treated food only.
  (3) "Clean" pen environment and food with treated water only.
  or(4) Other combinations.

  Birds in half the pens at a given treatment rate may be fasted and water withheld for 12 hours prior to the pesticide applications. If so, half the control pens should also be fasted and water withheld for the same 12-hour period.

  QUALITY CONTROL -- Toxicants should be carried in a table-grade corn oil.

  QUALITY CONTROL -- Food and water treatments should be made with procedures and rates that are consistent with the characteristic of the chemical and the usage being tested.

  QUALITY CONTROL -- Adequate replicates must be used if various treatment combinations are tested.

Conduct of Experiment

QUALITY CONTROL -- Establish regular audits of performance.

- Place pens, shelters and feeders in position, and introduce birds into pens as described above prior to the pesticide administration.

  QUALITY CONTROL -- All pens should be numbered and locations mapped or charted.

  QUALITY CONTROL -- All birds should be marked to facilitate accurate data collection.

- Administer the chemical as indicated above and as desired in various treatment combinations.

- Provide sufficient food and water to the test birds at all times, except during the specified periods of fasting and water withholding.

- Test Observations:

  Mortality. In case of bobwhite, if either member of test pair dies, the survivor should be removed and held for observation and a fresh pair placed in the test pen. All survivors are held for the observation of possible toxic signs.

  QUALITY CONTROL -- The same individual should be at the post to observe the toxic signs of the intoxicated birds.

Reproduction. Reproductive success of the test birds should be observed during the year of the test. Eggs may be picked up periodically for artificial incubation and rearing of young in the first half of the breeding season but eggs may be left for the hen(s) to incubate in the last half of the normal breeding season. Hens should be allowed to rear the young to 14 days of age in test pens.
Test duration. This should be a minimum of 21 days after the final pesticide application. It must be longer if any birds are showing toxic signs or other effects. Reproductive test would certainly continue beyond 21 days post-treatment.
Residue analysis. Confirm diet and water levels of the test chemicals. Analyze vegetation, soil and other environmental samples for residues in accordance with other label requirements and determine the persistence and bioaccumulation. Analyze the dead and surviving birds for residues in selected organs and/or tissues. Determine the gross pathology at the same time.
QUALITY CONTROL -- For pesticide residue analyses, use the two following standard manuals: (1) Manual of Analytical Methods for the Analysis of Pesticide Residues in Human and Environmental Samples. U.S. EPA, HERL-RTP, Environmental Toxicology Division, Rev. in June, 1974. (2) Manual of Analytical Quality Control for Pesticides in Human and Environmental Media. U.S. EPA, HERL-RTP, Environmental Toxicology Division. J.F. Thompson (ed.). EPA-600/1-76-017. February, 1977.
QUALITY CONTROL -- Train personnel for quality. See "Pesticide Residue Analysis in Water-Training Manual. U.S. Environmental Protection Agency, Office of Water Programs. EPA-430/1-74-012. September, 1974.

Collecting and Reporting of Data

- Collect the data on mortality - number, dates, etc.; toxic signs; weight changes; food consumption; clinical observations; necropsy observations; residue analysis results; weather data of tests; reproduction test(s) results.
  QUALITY CONTROL -- Signing and witnessing of data collection.
  QUALITY CONTROL -- Use statistical expertise in analysis of results.

- Report all the data collected above and test methods and materials used.
  QUALITY CONTROL -- Adopt a system for review and publication of data and reports.

Reference

- U.S. EPA, 1975. Guidelines for Registering Pesticides in the United States, Appendix, Part VII - Hazard Evaluation, Subpart C: Wildlife Toxicology. Federal Register, Vol. 40, No. 123 - Wednesday, June 25, 1975, pp. 26921-26922.

EXAMPLE 1: FULL-SCALE FIELD TESTS TO EVALUATE PESTICIDE HAZARDS TO WILDLIFE

Purpose of Study

- To determine the total impact of pesticide applications on wildlife populations.

Materials

- All wildlife including arthropods on sprayed and unsprayed (control) areas.

Design of Experiment

- A thorough pesticide-wildlife ecology study should include collection of data on wild birds and mammal populations (resident and nonresident species), climate, soil, vegetation biomass by species, numbers and biomass of arthropods, food habits of the most abundant wildlife species, and distribution and fate of pesticide residues in animals, plants, and environment. Each parameter would require a separate sampling method. These data should be collected on sprayed and unsprayed areas before and after the treatment dates.

Treatment Areas

- Treatment areas should be a minimum of 130 ha (320 acres) in size for a given chemical and rate of application. Cropland or right-of-way study areas may be smaller if the typical field or area sprayed would be smaller.
  QUALITY CONTROL -- All treatment areas should be sufficiently large to accommodate a minimum of 2 replicates of 8 to 16 ha (20 to 40 acres) census plots with a sprayed buffer zone of at least 45 m (150 ft) around all plot boundaries.

- The experimental applications should be made at the proposed registration rate and at two multiples of that rate, e.g., 3x or 5x.
  QUALITY CONTROL -- Control areas should be studied simultaneously on replicated plots in the same manner as the sprayed areas.

Conduct of Experiment

- Strip census is generally used for censusing cropland or rangeland birds. The basic procedure in this census technique is to walk a straight line transect, usually within a given time period, and identify, record and plot locations of all birds seen within a predetermined width of strip, e.g., 50 m to either side of the line to travel, i.e., a width of 100 m. Transects are marked in some manner so that the same routes can be repeated daily, weekly, monthly, seasonally or yearly.
  QUALITY CONTROL -- Strip censuses should be run in the early morning hours to coincide with a major activity period of the birds.

. . . .

QUALITY CONTROL -- Frequent counts within the breeding season on replicated transects will provide statistically adequate data for comparing pre- and postspray populations and sprayed plots with unsprayed plots.
QUALITY CONTROL -- Experienced personnel must go on the trip.

Plot census is usually used for censusing birds in forested or mixed habitats. The general approach for this census is similar to the strip census. The basic difference is that birds are observed, identified and plotted on a map of a square or rectangular plot, usually approximately about 16 ha (40 acres) in size. The observer walks a more or less fixed route taking him to all portions of the census plot within a given time period.
QUALITY CONTROL -- Same as for strip census.

Mark and recapture method for small mammals using grids of Sherman-type live traps: There are various systems of trap layouts, length of trapping period, and data treatment. The International Biological Program (IBP), Grassland Biome (Swift and French 1972) has recommended the system of utilizing a square grid of 12x12 stations (or 144 trap sites) (15 m between stations) with 1 or 2 live traps per station. Animals captured are marked and released over a trapping period of 5 consecutive days. Data are analyzed by the Jolly (1965) method.
QUALITY CONTROL -- The mark and recapture procedure must be repeated in the same manner in a pre- and postsprayed period on marked, replicated grids.
QUALITY CONTROL -- A control must be used.

The effects on target insects and total arthropod numbers and biomass should be measured by standard entomological methods. Particular attention should be paid to arthropod species known to be important for wildlife food.
QUALITY CONTROL -- The limitations of the arthropod sampling techniques used should be noted and reported.
QUALITY CONTROL -- Use an adequate manual for arthropod population analysis, e.g., Methods of Study in Quantitative Soil Ecology: Population, Production and Energy Flow (Phillipson, 1972).

Residue analyses should be done on the following types of samples:
- tissues of one or more species of common resident omnivorous mammals,
- tissues of one or more species of common resident omnivorous birds,
- common arthropods,
- vegetation including entire above-ground parts,
- plant litter,
- soil (to a depth of about 2½ cm),
- water (if any) from the sprayed area.

QUALITY CONTROL -- Use the best available technique for residue analysis in replicated aliquots.
QUALITY CONTROL -- Delayed analysis will invalidate the data.
QUALITY CONTROL -- All samples should be collected periodically in duplicates until residue levels fall below 0.01 ppm.

Data Collection and Handling

Collect the data on mortality with dates, signs of intoxication, wildlife census results, arthropod numbers and biomass, pathology, residue analysis, nest studies, weather conditions during the study period, fledgling observations, and other studies on reproduction of resident wildlife.
QUALITY CONTROL-- Signing and witnessing of data. Integrate all data into a picture of the total ecology of the introduction of the pesticide.
QUALITY CONTROL -- Use available statistical methods of analysis and statistical expertise in data analysis.

References:

The discussion here is principally derived from the following report: U.S. EPA, 1975. Guidelines for Registering Pesticides in the United States, Appendix, Part VII - Hazard Evaluation, Subpart C: Wildlife Toxicology. Federal Register, Vol. 40, No. 123 - Wednesday, June 25, 1975, pp. 26926-26928. Other references are:

Jolly, G.M 1965. Explicit estimates from capture-recapture data with both death and immigration-stochastic model. Biometrika. 52: 225-247.

Swift, D.M., and N.R. French. 1972. Vertebrates - small mammals. Pages 24-28 in: Basic Field Data Collection Procedures for the Grassland Biome. IBP, Nat. Res. Ecol. Lab., Ft. Collins, Colorado, 86 p. (Tech. Rpt. No. 145).

3.2.6 REFERENCES

Adams, E. S. 1975. Effect of lead and hydrocarbons from snowmobile exhaust on brown trout *Salvelinus fontinalis*. Trans. Amer. Fish. Soc. 104(2): 363-373.

Alabaster, J. S. 1969. Effects of heated discharges on freshwater fish in Britain. In: Biological Aspects of Thermal Pollution. P. A. Krenkel and F. L. Parker (eds.). Vanderbilt University Press. pp. 354-381.

Anderson, R. M. 1964. Methods of collecting and preserving vertebrate animals. 3rd Edition. National Museum of Canada, Bull. No. 69, Biol. Ser. No. 18.

Andrews, A. K., et al. 1966. Some effects of heptachlor on bluegills (*Lepomis macrochirus*). Trans. Amer. Fish. Soc. 95(3): 297-309.

Auclair, A. N. D., et al. 1976. Plant standing crop and productivity relations in a *Scirpus-Equisetum* wetland. Ecology 57: 941-952.

Blackburn, R. D., et al. 1968. Ecology of submerged aquatic weeds in South Florida canals. Weed Sci. 16: 261-266.

Bridges, W. R. 1958. Sodium Cyanide as a Fish Poison. U.S. Fish and Wildl. Serv. Sci. Rep. (Fish), No. 253. p. 11.

Burress, R. M. 1975. Development and Evaluation of On-site Toxicity Test Procedures for Fishery Investigations. U.S. Dept. Interior, Fish and Wildlife Service, Investigations in Fish Control No. 68, Washington, D.C.

Carlander, K. D. 1969. Handbook of Freshwater Fishery Biology. Vol. I. Iowa State Univ. Press, Ames, Iowa.

Carlson, A. R., and J. G. Hale. 1972. Successful spawning of largemouth bass *Micropterus salmoides* (Lacepede) under laboratory conditions. Trans. Amer. Fish. Soc. 101: 539.

Carpenter, K. E. 1925. On the biological factors involved in the destruction of river-fisheries by pollution due to lead-mining. Ann. Appl. Biol. 12: 1-13.

Castagna, M., and W. Duggan. 1971. Rearing the bay scallop, *Argopecten irradians*. Proc. Nat. Shellfish. Ass. 61: 80.

Cook, H. L. 1967. A method of rearing penaeid shrimp larvae for experimental studies. Food Agr. Organ. U.N., FAO Fish. Bull. 3: 709.

Coswell, B. C. 1965. The effect of sodium arsenite and silvex on the plankton populations in farm ponds. Trans. Amer. Fish. Soc. 94: 371-377.

Croker, R. A., and J. Wilson. 1965. Kinetics and effects of DDT in a tidal marsh ditch. Trans. Amer. Fish. Soc. 94: 152-159.

Dacre, J. C., and D. Scott. 1973. Effects of dieldrin on brown trout in field and laboratory studies. N. F. J. Mar. Freshwater Res. 7(3): 235-246.

Davis, L. V., and I. E. Gray. 1966. Distribution of insects in North Carolina Salt Marshes. Ecol. Monogr. 36(3): 275-295.

Eberhardt, L. L. 1971. Population analysis. In: Wildlife Management Techniques. R. H. Giles, Jr. (ed.). The Wildlife Society, Washington, D.C.

Edmondson, W. T., and G. G. Winberg (eds.). 1971. A Manual on Methods for the Assessment of Secondary Productivity in Fresh Waters. IBP Handbook No. 17, Blackwell Scientific Publications, Oxford and Edinburgh, England.

Edwards, R. W., and M. Owens. 1960. The effects of plants on river conditions. I. Summer crops and estimates of net productivity of macrophytes in a chalk stream. J. Ecol. 48: 151-160.

Eipper, A. W. 1959. Effects of five herbicides on farm pond plants and fish. N.Y. Fish Game J. 6: 46-56.

Falk, M. R. 1973. Simple apparatus for conducting acute toxicity bioassays under field conditions. Water Res. 7: 821-822.

Federal Water Pollution Control Administration. 1967. Temperature and Aquatic Life. Laboratory Investigation No. 6, Technical Advisory and Investigations Branch, Cincinnati, Ohio.

Forsberg, N. C. 1959. Quantitative sampling of subaquatic vegetation. Oikos 10: 233-240.

Fremling, C. R. 1967. Methods for mass-rearing Hexagenia (Ephemeroptera: Ephemeridae). Trans. Amer. Fish. Soc. 96: 407.

George, J. L., et al. 1957. Effects on wildlife of aerial applications of strobane, DDT, and BHC to tidal marshes in Delaware. J. Wildlife Manage. 21: 42-53.

Gerhold, R. M. 1973. Mobile bioassay laboratories. In: Biological Methods for the Assessment of Water Quality. American Society for Testing and Materials, ASTM STP 528, pp. 242-256.

Giles, R. H., Jr. (ed.). 1971. Wildlife Management Techniques. The Wildlife Society, Washington, D.C.

Gorbach, S. R., et al. 1971. Residue analyses and biotests in rice fields of East Java treated with thiodan. Bull. Environ. Contam. Toxicol. 6(2): 193-199.

Hansen, D. J., and P. R. Parrish. 1977. Suitability of sheepshead minnows (Cyprinodon variegatus) for life-cycle toxicity tests. In: Aquatic Toxicology and Hazard Evaluation. F. L. Mayer and J. L. Hamelink (eds.). ASTM STP 634.

Heinle, D. R. 1969. Culture of calanoid copepods in synthetic seawater. J. Fish. Res. Board Can. 26: 150.

Heitmuller, P. T., and D. R. Nimmo. 1972. A cage for exposing aquatic animals to bottom sediments. Progr. Fish-Cult. 34(2): 120.

Hemphill, J. E. 1954. Toxaphene as a fish toxin. Progr. Fish-Cult. 16(1): 41-42.

Hendrick, R. D., and T. R. Everett. 1965. Toxicity to the Louisiana red crawfish of some pesticides used in rice culture. J. Econ. Entomol. 58(5): 958-61.

Hirano, R., and Y. Oshima. 1963. Rearing of larvae of marine animals with special reference to their food organisms. Bull. Jap. Soc. Sci. Fish. 29: 282 (in Japanese).

Hoffman, D. A., and J. R. Olive. 1961. The effects of rotenone and toxaphene upon plankton of two Colorado reservoirs. Limnol. Oceanogr. 6(2): 219-222.

Hokanson, K. E. F., et al. 1973. Thermal requirements for maturation, spawning, and embryo survival of the brook trout, Salvelinus fontinalis. J. Fish. Res. Board Can. 30: 975.

Hughes, T. T., et al. 1974. A rearing tank for lobster larvae and other aquatic species. Progr. Fish-Cult. 36: 129.

Jervis, R. A. 1969. Primary production in the freshwater marsh ecosystems of Troy Meadows, New Jersey. Bull. Torrey Bot. Club 96: 209-231.

Kallman, B. J., O. B. Cope, and R. J. Navarre. 1962. Distribution and detoxification of toxaphene in Clayton Lake, New Mexico. Trans. Amer. Fish. Soc. 91(1): 14-22.

Koenig, C. C., R. J. Livingston, and C. R. Cripe. 1976. Blue crab mortality: interaction of temperature and DDT residues. Arch. Environ. Contam. Toxicol. 4(1): 119-128.

Lackey, J. B. 1938. The manipulation and counting of river plankton and changes in some organisms due to formalin preservation. Public Health Rep. (U.S.) 53: 2080.

Lauer, G. J. 1974. Design of a pesticide sampling program for the water environment; and: Collection and preservation of water, mud, fish, blood and other biological specimens for pesticide analysis. Both in: Pesticide Residue Analysis in Water -- Training Manual. U.S. Environmental Protection Agency, Office of Water Programs, EPA-430/1-74-012.

Lawrence, G. H. 1951. Taxonomy of Vascular Plants. Macmillan Publ. Co., New York, N.Y.

Lawrence, J. M. 1950. Toxicity of some new insecticides to several species of pond fish. Progr. Fish-Cult. 12: 141-150.

Lee, R. F., et al. 1972. Petroleum hydrocarbons - Uptake and discharge by the marine mussel, *Mytilus edulis*. Science 17(4046): 344-46.

Livingston, R. J. (ed.), et al. 1974. Synergism and Modifying Effects: Interacting Factors in Bioassay in Field Research. Spec. Publ., Marine Tech. Soc.

Loosanoff, V. L., and H. C. Davis. 1963. Rearing of bivalve mollusks. Advan. Mar. Biol. 1: 1.

Ludwig, P. D., et al. 1968. Biological effects and persistence of dursban insecticide in a salt-marsh habitat. J. Econ. Entomol. 61: 626-633.

Maddux, W. S., and J. W. Kanwisher. 1965. An *in situ* particle counter. Limnol. Oceanogr. 10(Suppl.): R 162.

May, B. E., R. S. Hestand, and J. M. Van Dyke. 1973. Comparative effects of diquat plus copper sulfate on aquatic organisms. Weed Sci. 21(3): 249-53.

May, R. C. 1970. Feeding larval marine fishes in the laboratory: A review. Calif. Mar. Res. Comm. CALCOFI Rept. 14: 76.

Mayhew, J. 1959. The use of toxaphene as a fish poison in strip mine ponds with varying physical and chemical characteristics. Proc. Iowa Acad. Sci. 66: 513-517.

McCormick, J. H., et al. 1972. Effects of temperature on growth and survival of young brook trout, *Salvelinus fontinalis*. J. Fish. Res. Board Can. 29: 1107.

McFarren, E. F. 1974. Sampling for pesticide residue analysis. Outline No. 20 in: Pesticide Residue Analysis in Water -- Training Manual. U.S. Environmental Protection Agency, Office of Water Programs, EPA-430/1-74-012.

Middaugh, D. P., and J. M. Dean. 1974. The Toxicity of Cadmium to the Eggs, Larvae and Adults of the Mummichog, *Fundulus heteroclitus*, and the Silverside, *Menidia Menidia*. Bears Bluff Field Station, U.S. Environmental Protection Agency.

Middaugh, D. P., and P. W. Lempesis. 1976. Laboratory spawning and rearing of a marine fish, the silverside *Menidia menidia menidia*. Mar. Biol. 35: 295-300.

Milner, C., and R. E. Hughes. 1968 (reprinted 1970). Methods for the Measurement of the Primary Production of Grassland. IBP Handbook No. 6, Blackwell Scientific Publications, Oxford and Edinburgh, England.

Mock, C. R. 1974. Larval Culture of Penaeid Shrimp at the Galveston Biological Laboratory. NOAA Tech. Rep. NMFS Circ. 388: 33.

Mosby, H. S., and I. M. Cowan. 1971. Collection and field preservation of biological materials. In: Wildlife Management Techniques. R. H. Giles, Jr. (ed.). The Wildlife Society, Washington, D.C. pp. 259-275.

Moulton, T. P. 1974. The effects of various insecticides (especially thiodan and BHC) on fish in the paddy-fields of West Malaysia. Malaysian Agr. J. 49(2): 224-253.

Moye, W. C., and W. H. Luckmann. 1964. Fluctuations in population of certain aquatic insects following application of aldrin granules to Sugar Creek, Iroquois County, Illinois. J. Econ. Entomol. 57(3): 318-322.

Mulla, M. S. 1962a. Frog and toad control with insecticides. Pest Contr. 30(10): 20.

Mulla, M. S. 1962b. Toxicity of organochlorine insecticides to the mosquitofish and the bullfrog. Mosquito News 23(4): 299-303.

Mullin, M. M., and E. R. Brooks. 1967. Laboratory culture, growth rate and feeding behavior of a planktonic marine copepod. J. Amer. Soc. Limnol. Oceanogr. 12: 657.

Nacht, L., and C. I. Weber. 1976. Biological Data Storage and Retrieval System (BIO-STORET). U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio.

Nakatani, R. E. 1969. Effects of heated discharges on anadromous fishes. In: Biological Aspects of Thermal Pollution. P. A. Krenkel and F. L. Parker (eds.). Vanderbilt University Press. pp. 294-353.

NAS. 1973. Nutrient Requirements of Trout, Salmon and Catfish. 11: 1. Publ. Office, National Academy of Sciences, Washington, D.C.

NAS. 1974. Water Quality Criteria, 1972. A report of the Committee on Water Quality Criteria. National Academy of Sciences. U.S. Government Printing Office, Washington, D.C.

Needham, J. G., et al. 1937. Culture Methods for Invertebrate Animals. Comstock Publishing Co., reprinted by Dover Publ., Inc., New York, N.Y.

Nolan, M. O., and E. G. Berry. 1949. Preliminary field trials with laboratory-tested molluscacides. Public Health Rep. (U.S.) 64(30): 942-949.

Odum, E. P. 1971. Fundamentals of Ecology. 3rd Edition. W. B. Saunders Co., Philadelphia, London, Toronto.

Overton, W. S. 1971. Estimating the numbers of animals in wildlife populations. In: Wildlife Management Techniques. R. H. Giles, Jr. (ed.). The Wildlife Society, Washington, D.C. pp. 403-455.

Parker, B. L., and J. E. Dewey. 1969. Further improvements on the mass rearing of Daphnia magna. J. Econ. Entomol. 62: 725.

Perkins, E. J. 1972. Some problems of marine toxicity studies. Mar. Pollut. Bull. 3: 13-15.

Petrusewicz, K., and A. Macfadyen. 1970. Productivity of Terrestrial Animals - Principles and Methods. IBP Handbook No. 13. Blackwell Scientific Publications, Oxford and Edinburgh, England.

Phillipson, J. 1972. Methods of Study in Quantitative Soil Ecology: Population, Production and Energy Flow. IBP Handbook No. 18, J. B. Lippincott Co., Philadelphia, Pa.

Prater, B. L., and M. A. Anderson. 1976. A mass-rearing method for burrowing mayflies. In: Analytical Quality Control Newsletter 30, July 1976. U.S. Environmental Protection Agency, Cincinnati, Ohio.

Rand, M. C., A. E. Greenberg, and M. J. Taras. 1975. Standard Methods for the Examination of Water and Wastewater. 14th Edition. American Public Health Association, American Water Works Association, and Water Pollution Control Federation, Washington, D.C.

Rawls, C. K. 1965. Field tests of herbicide toxicity to certain estuarine animals. Chesapeake Sci. 6: 150-161.

Ricker, W. E. (ed.). 1971. Methods for Assessment of Fish Production in Fresh Waters. 2nd Ed. IBP Handbook No. 3, Blackwell Scientific Publications, Oxford and Edingburgh, England.

Siefert, R. E. 1972. First food of larval yellow perch, white sucker, bluegill, emerald shiner and rainbow smelt. Trans. Amer. Fish. Soc. 101: 219.

Slack, K. V., et al. 1973. Methods for collection and analysis of aquatic biological and microbiological samples. In: Techniques of Water Resources Investigations of the United States Geological Survey. Book 5, Chapter A4.

Sladeckova, A. 1962. Limnological investigation methods for the periphyton ("Aufwuchs") community. Bot. Rev. 28: 286-350.

Smith, R. A., et al. 1974. Automatic brine shrimp feeder. Progr. Fish-Cult. 36: 133.

Spotte, S. H. 1970. Fish and Invertebrate Culture Water Management in Closed Systems. John Wiley and Sons, Inc., New York, N.Y.

Sprague, J. B., P. F. Elson, and R. L. Saunders. 1965. Sublethal copper-zinc pollution in a salmon river - a field and laboratory study. Int. J. Water Pollut. 9(9): 531-43.

Springer, P. F., and J. R. Webster. 1951. Biological effects of DDT applications on tidal salt marshes. Mosquito News 11: 67-74.

Stalnaker, C. B., and R. E. Gresswell. 1974. Early Life History and Feeding of Young Mountain White Fish. U.S. Environmental Protection Agency, Office of Research and Development, EPA-660/3-73-019, Washington, D.C.

Stewart, W. D. P., et al. 1967. In situ studies on N_2 fixation using acetylene reduction technique. Proc. Nat. Acad. Sci. U.S.A. 58: 2071.

Stewart, W. D. P., et al. 1970. Acetylene reduction assays for determination of phosphorus availability in Wisconsin Lakes. Proc. Nat. Acad. Sci. U.S.A. 66: 1104.

Strickland, J. D. H., and T. R. Parsons. 1968. A Practical Handbook of Seawater Analysis. Fish. Res. Board Can., Bull. No. 167, 311 pp.

Summerfelt, R. C., and W. M. Lewis. 1967. Repulsion of green sunfish by certain chemicals. J. Water Pollut. Contr. Fed. 39(12): 2030-2038.

Swartz, R. C. 1976. Research needs concerning pollution of the marine benthos. In: Proceedings of the International Symposium on Marine Pollution Research, Jan. 27-29, 1976. S.P. Meyers (ed.). Center for Wetland Resources, Louisiana State University, Baton Rouge, La.

Taber, R. D. 1971. Criteria of sex and age. In: Wildlife Management Techniques. R. H. Giles, Jr. (ed.). The Wildlife Society, Washington, D.C. pp. 325-401.

Tagatz, M. E., et al. 1974. Effects of ground application of malathion on saltmarsh environment in north-western Florida. Mosquito News 34: 199-206.

Tarzwell, C. M. 1948. Effects of routine DDT mosquito larviciding on wildlife. J. Nat. Malar. Soc. 7: 199-206.

Unrau, G. D., et al. 1965. Field trials in Egypt with acrolein herbicide-molluscide. Bull. World Health Organ. 32: 249-260.

U.S. DHEW. 1974. Guide for the Care and Use of Laboratory Animals. U.S. Department of Health, Education and Welfare, NIH 74-23.

U.S.D.I. 1967. Pesticide Surveillance Program, Effects of Granular Application of Dursban on Some Estuary and Salt Marsh Organisms. Special Report, Fish and Wildlife Services. U.S. Dept. of the Interior.

U.S.D.I. 1968. Pesticide Field Appraisal, Field Appraisal of Tests to Control Salt Marsh Mosquitos with Dursban Applied as a Larvicide and Adulticide in Florida. Special Report, Fish and Wildlife Services. U.S. Department of the Interior.

U.S.D.I. 1972. Recommended Methods for the Acquisition of Data. Chapter IV. On the biologic, bacteriologic and chemical (organic) quality of water. Prepared by the Task Group on Biologic Quality and Organics of the Federal Interagency Work Group on Designation of Standards for Water Data Acquisition. U.S. Department of the Interior.

U.S. EPA. 1972. Handbook for Analytical Quality Control in Water and Wastewater Laboratories. U.S. Environmental Protection Agency, Analytical Quality Control Laboratory, Cincinnati, Ohio.

U.S. EPA. 1973. Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents. C. I. Weber (ed.). U.S. Environmental Protection Agency, EPA-670/4-73-001, Cincinnati, Ohio.

U.S. EPA. Region II. 1975. Quality Assurance Manual - Biology Section. U.S. Environmental Protection Agency, Region II, Edison, N.J.

U.S. EPA. 1975a. Hazard Evaluation, Aquatic Toxicology--Field Testing Techniques. U.S. Environmental Protection Agency. Fed. Regist. 40(123): 26909-26912.

U.S. EPA. 1975b. Hazard Evaluation, Wildlife Toxicology. U.S. Environmental Protection Agency. Fed. Regist. 40(123): 26912-26928.

U.S. EPA. 1976a. Handbook for Sampling and Sample Preservation of Water and Wastewater. U.S. Environmental Protection Agency, EPA-600/4-76-049, Cincinnati, Ohio.

U.S. EPA. 1976b. Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Environmental Research Laboratory, EPA-600/9-76-010, Gulf Breeze, Fla.

U.S. EPA. 1976c. Analytical Quality Control Newsletter 31, October, 1976. U.S. Environmental Protection Agency, Cincinnati, Ohio.

U.S. EPA. 1976d. Quality Criteria for Water. U.S. Environmental Protection Agency, EPA-440/9-76-023, Washington, D.C.

U.S. EPA. 1977. Analytical Quality Control Newsletter 33, April, 1977. U.S. Environmental Protection Agency, Cincinnati, Ohio.

Vermeer, K., et al. 1974. Pesticide effects on fishes and birds in rice fields of Surinam, South America. Environ. Pollut. 7(3): 217-236.

Weber, C. I. 1971. A Guide to the Common Diatoms at Water Pollution Surveillance System Stations. U.S. Environmental Protection Agency, National Environemental Research Center, Cincinnati, Ohio.

Weber, C. I. 1973. Recent developments in the measurement of the response of plankton and periphyton to changes in their environment. In: Bioassay Techniques and Environmental Chemistry. G. Glass (ed.). Ann Arbor Sci. Publ., Inc., Ann Arbor, Mich.

Weber, C. I. 1975. Evaluation of Aquatic Biology Programs, Parts 1 to 3. U.S. Environmental Protection Agency, Cincinnati, Ohio.

Weber, C. I. 1976. Bio-Storet Master Species List, Parts 1 and 2. U.S. Environmental Protection Agency, Cincinnati, Ohio.

Weber, C. I. 1977. Personal communication.

Westlake, D. F. 1966. The biomass and productivity of Glyceria Maxima. I. Seasonal changes in biomass. J. Ecol. 54: 745-753.

Westlake, D. F. 1968. Methods used to determine the annual production of reedswamp plants with extensive rhizomes. In: Methods of Productivity Studies in Rootsystems and Rhizosphere Organisms. IBP Root Symposium, Moscow, 1968, 226-234. NAUKA, Moscow, USSR.

Westlake, D. F. 1974. Macrophytes. In: A Manual on Methods for Measuring Production in Aquatic Environments. R. A. Vollenweider (ed.). IBP Handbook No. 12, pp. 32-42. Blackwell Scientific Publications, Oxford and Edinburgh, England.

Wetzel, R. G., and D. F. Westlake. 1974. Perpiphyton. In: A Manual on Methods for Measuring Primary Production in Aquatic Environments. R. A. Vollenweider (ed.). IBP Handbook No. 12, pp. 42-50. Blackwell Scientific Publications, Oxford and Edinburgh, England.

Workman, G. W., and J. M. Newhold. 1963. Lethal concentrations of toxaphene for goldfish, mosquito fish, and rainbow trout, with notes on detoxification. Progr. Fish-Cult. 25(1): 23-30.

Zillich, J. 1969. The simultaneous use of continuous flow bioassays and automatic water quality monitoring equipment to evaluate the toxicity of waste water discharges. Presented at the 44th Annual Conf. of Mich. Water Poll. Cont. Assoc., Boyne Falls, Mich., June 16, 1969.

Zillioux, E. J., and D. F. Wilson. 1966. Culture of a planktonic calanoid copepod through multiple generations. Science 151: 996.

General Bibliography on Identification of Organisms for Bioassay Tests

Viruses

Andrewes, C. H., and H. G. Pereira. 1972. Viruses of Vertebrates. 3rd Ed., Bailliere, Tindall and Cox, London, England.

Gibbs, A. J., et al. 1966. What's in a virus name? Nature 209: 450.

Melnick, J. L. 1971, 1972. Classification and nomenclature of animal viruses, 1971 and 1972. Progr. Med. Virol. 13: 462-484, 1971; 14: 321-332, 1972.

Newman, J. F. E., et al. 1973. A physico-chemical sub-grouping of the mammalian picornaviruses. J. Gen. Virol. 18: 171.

Rosen, L., et al. 1970. Subclassification of enteroviruses and ECHO virus type 34. Arch. Gesam. Virusforsch. 30: 39.

Wildy, P. 1971. Classification and nomenclature of viruses. International Committee on Nomenclature of Viruses. Monogr. Virol. 5: 1.

World Health Organization. 1971. A revised system of nomenclature for influenza viruses. Bull. World Health Organ. 45: 119.

Fungi

Barnett, H. L. 1960. Illustrated Genera of Imperfect Fungi. Burgess Publishing Company, Minneapolis, Minn.

Barron, G. L. 1968. The Genera of Hyphomycetes from Soil. Williams and Wilkins, Baltimore, Md.

Cooke, W. B. 1963. A Laboratory Guide to Fungi in Polluted Waters, Sewage, and Sewage Treatment Systems. Their Identification and Culture. U.S. Public Health Service, Publ. 999-WP-1, Cincinnati, Ohio.

Gillman, J. C. 1957. Manual of Soil Fungi. Iowa State University Press, Ames, Iowa.

Grove, W. B. 1937. British Stem and Leaf Fungi. University Press, Cambridge, England.

Raper, K. B., and D. I. Fennel. 1965. The Genus Aspergillus. Williams and Wilkins, Baltimore, Md.

Bacteria and Actinomycetes

Breed, R. S., et al. 1957. Bergey's Manual of Determinative Bacteriology. 8th Ed. Bailliere, Tindall and Cox, London, England.

Buchanan, R. E., et al. 1966. Index Bergeyana. E. & S. Livingston, Edinburgh and London, England.

Cowan, S. T., and K. J. Steel. 1961. Diagnostic tables for the common medical bacteria. J. Hyg. 59: 357.

Cowan, S. T., and K. J. Steel. 1965. Manual for the Identification of Medical Bacteria. University Press, Cambridge, England.

Gordan, R. E. 1968. The taxonomy of soil bacteria. In: The Ecology of Soil Bacteria. T. R. G. Gray and D. L. Parkinson (eds.). University Press, Liverpool, England.

Skerman, V. B. D. 1967. A Guide to the Identification of the Genera of Bacteria. 2nd Ed. Williams and Wilkins, Baltimore, Md.

Smith, N. R., et al. 1952. Aerobic Spore-forming Bacteria. U.S. Department of Agriculture, Monograph 16, Washington, D.C.

Williams, S. T., et al. 1968. Identification of genera of the Actinomycetales. In: Identification Methods for Microbiologists. B. M. Gibbs and D. A. Shapton (eds.). Academic Press, London, New York.

Protozoa

Bick, H. 1967-69. An Illustrated Guide to Ciliated Protozoa (used as biological indicators in freshwater ecology). Parts 1-9. World Health Organization, Geneva, Switzerland.

Kudo, R. R. 1963. Protozoology. Charles Thomas, Springfield, Ill.

Other Microinvertebrates

Donner, J. 1966. Rotifers. Butler and Tanner, Ltd., London, England.

Edmundson, W. T. 1959. Freshwater Biology. John Wiley and Sons, New York, N.Y.

Pennak, R. W. 1953. Freshwater Invertebrates of the United States. Ronald Press, New York, N.Y.

Fishes

See Section 6.4, Fish Identification, in: Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents (U.S. EPA, 1973). This section gives general references for fish identification, as well as references for regional fish identifications, such as:

- Marine: Costal Pacific
- Marine: Atlantic and Gulf of Mexico
- Freshwater: Northeast
- Freshwater: Southeast

- Freshwater: Midwest
- Freshwater: Southwest
- Freshwater: Northwest.

Amphibia

Bishop, S. C. 1943. Handbook of Salamanders. Comstock Publishing Company, Ithaca, N.Y.

Conant, R. 1958. A Field Guide to Reptiles and Amphibians of Eastern North America. Houghton Mifflin Company, Boston, Mass.

Cochran, D. M. 1961. Living Amphibians of the World. Doubleday & Company, New York, N.Y.

Dunn, E. R. 1926. The Salamanders of the Family Plethodontidae. Smith College, Northampton, Maine.

Noble, G. K. 1931. Biology of the Amphibia. McGraw-Hill Book Company, Inc., New York, N.Y.

Stebbins, R. C. 1951. Amphibians of Western North America. University of California Press, Berkeley, Calif.

Wright, A. H., and A. A. Wright. 1949. Handbook of Frogs and Toads of the United States and Canada. Comstock Publishing Company, Ithaca, N.Y.

Reptilia

Barbour, T. 1934. Reptiles and Amphibians. 2nd Ed. Houghton Mifflin Co., Boston, Mass.

Carr, A. F., Jr. 1952. Handbook of Turtles. Cornell University Press, Ithaca, N.Y.

Conant, R. 1951. The Reptiles of Ohio. 2nd Ed. University of Notre Dame, Notre Dame, Ind.

Ditmars, R. L. 1944. Snakes of the World. Macmillan Publ. Co., New York, N.Y.

Gadow, H. 1901. Amphibia and Reptiles (Cambridge Natural History). MacMillan & Co., London, England.

Goin, C. J., and O. B. Goin. 1962. Introduction to Herpetology. W. H. Freeman & Co., Publ., San Francisco, Calif.

Klauber, L. M. 1956. Rattlesnakes, 2 vols. University of California Press, Berkeley, Calif.

Pope, C. H. 1955. The Reptile World. Alfred A. Knopf, Inc., New York, N.Y.

Schmidt, K. P., and D. D. Davis. 1941. Field Book of Snakes of the United States and Canada. G. P. Putnam's Sons, New York, N.Y.

Smith, H. M. 1946. Handbook of Lizards of the United States and Canada. Comstock Publ. Co., Ithaca, N.Y.

Wright, A. M., and A. A. Wright. 1957. Handbook of Snakes of the United States and Canada, 2 vols. Comstock Publishing Co., Ithaca, N.Y.

Birds

Allen, A. A. 1936. Ornithology Laboratory Notebook. Comstock Publishing Co., Ithaca, N.Y.

American Ornithologists' Union. 1957. Check List of North American Birds. 5th Ed. Amer. Ornith. Union, Baltimore, Md.

Audubon, J. J. 1937. The Birds of America. Macmillan Publ. Co., New York, N.Y.

Bent, A. C. 1961-68. Life Histories of North American Birds. Dover Publications, New York, N.Y.

Forbush, E. H. 1939. Natural History of the Birds of Eastern and Central North America. Houghton Mifflin Co., Boston, Mass.

Gilliard, E. T. 1958. Living Birds of the World. Doubleday & Co., Inc., New York, N.Y.

Peters, J. L. 1931. Checklist of Birds of the World. Harvard University Press, Cambridge, Mass.

Peterson, R. T. 1947. Field Guide to the Birds. 2nd Ed. Houghton Mifflin Co., Boston, Mass.

Peterson, R. T. 1961. A Field Guide to Western Birds. Revised Ed. Houghton Mifflin Co., Boston, Mass.

Pettingill, O. S., Jr. 1956. A Laboratory and Field Manual of Ornithology. 3rd Ed. Burgess Publishing Co., Minneapolis, Minn.

Rand, A. L. 1956. American Water and Game Birds. E. P. Dutton & Co., New York, N.Y.

Van Tyne, J., and A. J. Berger. 1971. Fundamentals of Ornithology. Dover Publications, New York, N.Y.

Wood, N. A. 1951. The Birds of Michigan. University of Michigan Press, Ann Arbor, Mich.

Mammals

Anthony, H. E. 1928. Field Book of North American Mammals. G. P. Putnam's Sons, New York, N.Y.

Blair, W. F., et al. 1957. Vertebrates of the United States. McGraw-Hill Book Co., Inc., New York, N.Y.

Burt, W. H., and R. P. Grossenheider. 1952. A Field Guide to the Mammals. Houghton Mifflin Co., Boston, Mass.

Burt, W. H. 1957. Mammals of the Great Lakes Region. University of Michigan Press, Ann Arbor, Mich.

Cockrum, E. L. 1955. Manual of Mammalogy. Burgess Publishing Co., Minneapolis, Minn.

Collins, H. H. 1959. Complete Field Guide to American Wildlife. Harper & Brothers, New York, N.Y.

Davis, E. E., and F. B. Golley. 1964. Principles of Mammalogy. Reinhold Publishing Corporation, New York, N.Y.

Hall, E. R., and K. R. Kelson. 1959. The Mammals of North America, 2 vols. The Ronald Press Co., New York, N.Y.

Hamilton, W. J., Jr. 1939. American Mammals. McGraw-Hill Book Co., Inc., New York, N.Y.

Hartman, C. G. 1952. Possums. University of Texas Press, Austin, Tex.

Leopold, A. S. 1959. Wildlife of Mexico. University of California Press, Berkeley, Calif.

Littlewood, C. 1970. World's Vanishing Animals: The Mammals. Arco Publ. Co., New York, N.Y.

Sanderson, I. T. 1955. Living Mammals of the World. Doubleday & Co., Inc., New York, N.Y.

Scheffer, V. B. 1958. Seals, Sea Lions, and Walruses. A Review of the Pennipedia. Stanford University Press, Stanford, Calif.

Schmidt-Nielson, K. 1964. Desert Animals. Clarendon Press, Oxford, England.

Simpson, G. G. 1945. The principles of classification and a classification of mammals. Bull. Amer. Mus. Nat. Hist. 85: 1-350.

Tomich, P. Q. 1969. Mammals of Hawaii - A Synopsis and Notational Bibliography. Bernice P. Bishop Mus. Spec. Publ. 57, Bishop Mus. Press, Honolulu, Hawaii.

Vaughan, T. A. 1972. Mammalogy. W. B. Saunders Co., Philadelphia, London and Toronto.

Walker, E. P. 1968. Mammals of the World, 2 vols. 2nd Ed. Johns Hopkins Press, Baltimore, Md.

Plants

Britton, N. L. 1907. Manual of the Flora of the Northern States and Canada. 3rd Ed., revised and enlarged. New York, N.Y.

Britton, N. L., and A. Brown. 1913. An Illustrated Flora of the Northern United States, Canada, and British Possessions from Newfoundland to the Parallel of the Southern Boundary of Southern Virginia and from the Atlantic Westward to the 102nd Meridian. 2nd Ed., revised and enlarged. 3 vol. New York, N.Y.

Coulter, J. M. 1909. New Manual of Botany of the Central Rocky Mountains (Vascular Plants), revised by Aven Nelson. New York.

Dean, C. C. 1940. Flora of Indiana. Indianapolis.

Fernald, M. L. 1950. Gray's Manual of Botany, 8th Ed. Boston and New York.

Jepson, W. L. 1923-1925. A Manual of the Flowering Plants of California. Berkeley.

Jepson, W. L. 1909-1943. A Flora of California. 3 vols (incomplete). San Francisco, Berkeley.

Kearney, T. H., and R. H. Peebles. 1942. Flowering Plants and Ferns of Arizona. U.S. Dept. Agr. Misc. Publ. 423.

Munz, P. A. 1935. A Manual of Southern California Botany. Claremont, Calif.

Peck, M. E. 1941. A Manual of the Higher Plants of Oregon. Portland, Oregon.

Piper, C. V. 1906. Flora of the State of Washington. Contr. U.S. Nat. Herb., vol. 11.

Reeve, R. G., and D. C. Bain. 1946. A Flora of South Central Texas. College Station, Texas.

Rydberg, P. A. 1922. Flora of the Rocky Mountains and Adjacent Plains, Colorado, Utah, Wyoming, Idaho, Montana, Saskatchewan, Alberta and Neighboring Parts of Nebraska, South Dakota, North Dakota and British Columbia. 2nd Ed. New York.

Rydberg, P. A. 1932. Flora of the Prairies and Plains of Central North America. New York.

Schaffner, J. H. 1928. Field Manual of the Flora of Ohio and Adjacent Territory. Columbus.

Small, J. K. 1933. Manual of the Southern Flora, Giving Descriptions of the Seed Plants Growing Naturally in Florida, Alabama, Mississippi, Eastern Louisiana, Tennessee, North Carolina, South Carolina, and Georgia. New York.

Steman, T. R., and W. S. Myers. 1937. Oklahoma Flora. Oklahoma City.

Tidestrom, I. 1925. Flora of Utah and Nevada. Contr. U.S. Nat. Herb., vol. 25. p. 665.

Wooton, E. O., and P. C. Stanley. 1915. Flora of New Mexico. Contr. U.S. Nat. Herb., vol. 19.

Aquatic Plants

Beal, E. O., and P. H. Monson. 1954. Marsh and aquatic angiosperms of Iowa. State University, Iowa. Studies Nat. Hist. 19(5): 1-195.

Correll, D. S., and H. B. Correll. 1973. Aquatic and Wetland Plants of the Southwestern United States. Washington, D.C.

Eyles, D. E., and J. L. Robertson, Jr. 1944. A Guide and Key to the Aquatic Plants of the Southeastern United States. Public Health Bull. No. 286., U.S. Gov. Printing Office, Washington, D.C.

Fassett, N. C. 1967. A Manual of Aquatic Plants. 2nd Ed. Madison, Wis.

Hotchkiss, N. 1972. Common Marsh, Underwater and Floating-leaved Plants of the United States and Canada. New York, N.Y.

Mason, H. L. 1957. A Flora of the Marshes of California. Berkeley and Los Angeles, Calif.

Mitchell, D. S. (ed.). 1974. Aquatic vegetation and its use and control. A contribution to the Intern. Hydrol. Decade. UNESCO-Paris: pp. 1-135. ISBN 92-3-101082-4. Printed in France.

Muenscher, W. C. 1944. Aquatic Plants of the United States. Comstock Publishing Company, Ithaca, N.Y.

Otto, N. E., and T. R. Bartley. 1965. Aquatic Pests on Irrigation Systems, Identification Guide. Washington, D.C.

Prescott, G. W. 1969. The Aquatic Plants. Dubuque, Iowa.

Stewart, A. N., J. D. LaRea, and H. M. Gilkey. 1963. Aquatic Plants of the Pacific Northwest Oregon. 2nd Ed.

Stodola, J. 1967. Encyclopedia of Water Plants. New Jersey.

Winterringer, G. S., and A. C. Lopinot. 1966. Aquatic Plants of Illinois. III. State Museum Popular Ser. Vol. VI, ILL. State Museum Division.

3.3 AQUATIC BIOASSAY

3.3.1 Basic Requirements of Aquatic Bioassay

3.3.1.1 Personnel--

Almost without exception, activities in both experimental and applied toxicology have biological, chemical, and statistical aspects. Because of the interdisciplinary character of toxicological activities and because few people are really competent in more than one field, biologists, chemists, and statisticians must work together (Stephan, 1973). In aquatic biological laboratories the discipline of toxicology may also be represented but in smaller laboratories in particular, the toxicological aspects may be handled by the biologist with the support of the chemist.

The biologist, in any event, is required to maintain a broad overview of the basic toxicological questions and guides the whole experimental effort.

Chemists may be full members of the biological team or may play a supporting role by supplying analytical laboratory capability for one or more teams. The chemist can contribute to experimental toxicology by:

- aiding in the selection of toxicants that should be tested
- helping design toxicity tests
- measuring and characterizing the level of the toxicant to which the subjects are actually exposed
- determining the fate of the toxicant after it comes in contact with the subjects
- helping determine the mode of action of the toxicant
- aiding in detecting some of the effects of the toxicant on the subjects
- recommending good sample collection and dosage techniques
- devising ways to prepare special materials and toxic agents that have been designed by toxicologists.

Chemists are in a good position to identify actual and potential environmental contaminants because many of these are used or produced by the chemical industry.

Statisticians usually play a supporting role by:

- aiding in the design of bioassay
- providing good sampling plans
- helping ensure the validity of chemical and biological test results by calling for duplicate samples, standard samples, and interlaboratory samples
- suggesting methods for data analysis and assisting in the analysis and interpretation of data.

Fish bioassay techniques usually involve exposure of the experimental species to toxic agents in water rather than the direct application of agents to the animals. Thus, information regarding the chemical reaction between the toxic agent and the media (water) is very essential for designing and interpreting of toxicity tests conducted on fish.

Generally, biological tests will be better measures of biological properties than chemical tests, even if the biological tests are not as well developed as many of the chemical tests. Environmental protection needs toxicological accuracy as much as it needs statistical precision.

Training is available in the form of courses provided at Federal or Academic institutions. Laboratory personnel should be encouraged to attend professional meetings to help the individual keep abreast of the state of the art within his particular professional interest. Overall the biology laboratory as a unit benefits from individual training and self-enrichment programs (U.S. EPA, 1975b).

3.3.1.2 Facilities and Equipment--

- Facilities

For maximum convenience and versatility, the facilities should include:

- tanks for holding and acclimating test organisms
- a constant temperature area or recirculating water bath for the test chambers
- a dilution water tank that may be used to prepare reconstituted water and which is elevated, if possible, so dilution water can flow by gravity into holding and acclimation tanks and test chambers.

Ceilings should be at least 10 feet high to accommodate proportional diluters and strainers, and air traps should be included in the water supply system. Holding, acclimation, and dilution water tanks should be equipped for temperature control and aeration. The test facilities should be well ventilated and free of fumes (U.S. EPA, 1975a).

- Construction material

Construction materials and commercially purchased equipment that may contact any water into which test organisms are placed should not contain any substances that can be leached or dissolved by the water. In addition, materials and equipment that contact stock solutions or test solutions should be chosen to minimize sorption of toxicants from water. Glass, #316 stainless steel, and fluorocarbon plastics must be used whenever possible. Rubber, copper, brass and lead must not come in contact with dilution water, effluent samples, or test solutions (U.S. EPA, 1975a).

- Test chambers

Test chambers can be made by welding, not soldering, stainless steel or by gluing double-strength or stronger window glass with clear silicone adhesive. As little of adhesive as possible should be in contact with water; extra beads of adhesive should be on the outside of the chamber rather than on the inside (U.S. EPA, 1975a).

- Embryo and fry chambers

Embryo and fry chambers should be constructed to allow for adequate exchange of water and to ensure that the proper quantity of test material is entering the chambers. These chambers must be brushed daily to prevent clogging. Embryo and fry chambers should be designed so that water can be drained down to 2.5 cm (1 inch) in order to facilitate growth measurements of fry. These chambers may be supplied with the test water by:

o separate delivery tubes from the mixing chamber,
o splitting the flow from the aquaria,
o or "egg" cups on a "rocker" arm (U.S. EPA, 1976).

- Toxicant mixing chambers

A mixing chamber is necessary to assure adequate mixing of the test material. Aeration should not be used for mixing. Mixing is extremely important because if the test materials are not adequately mixed with water, toxicity cannot be properly assessed. Improper mixing can either expose the animal to too much or too little of the material, and toxicity would be over- or underestimated (ASTM, 1974).

- Calibration and standardization of test containers

Before filling the test containers, it is necessary to determine a suitable aeration rate so that the loss of any dissolved volatile substances from the liquid in the test container will be excessive. This involves determining the total number of bubbles of air or oxygen or both released per minute in a given test container filled with the test solution up to a given level. The dissolved oxygen content of the test solution shall not fall below 4 ppm when warm-water fish are used as test animals, or below 5ppm when cold-water fish are used and it should not exceed the saturation value at the experimental temperature.

Calibration method is as follows:

Fill the test container to the fixed level with clean soft water having an alkalinity to methyl orange indicator not in excess of 40 ppm as $CaCO_3$. Dissolve CO_2 gas in the water to obtain a concentration rate (in terms of the number of bubbles of air or oxygen released per minute) such that the amount of CO_2 lost from the solution in 24 hours under these experimental conditions will not exceed 67 percent of the initial free CO_2 (ASTM, 1974a)

- Toxicant delivery system

Although many toxicant delivery systems can be used (Lowe, 1964: Sprague, 1969; Freeman, 1971; Cline and Post, 1972; Granmo and Kollberg, 1972; Bengtsson, 1972; Lichatowich et al., 1973; Shumway and Plaensky, 1973; Abram, 1973; Schimmel et al., 1974; DeFoe, 1975; National Water Quality Laboratory, Duluth, Minnesota, personal communication; Garton, R., Western Fish Toxicology Station, Corvallis, Oregon, personal communication), the proportional diluter (Mount and Brungs, 1967) is considered to be the best for routine use. One disadvantage of the Mount and Brungs diluter is that it is impractical when the dilution factors between concentrations exceed fifty percent and the logarithmic gradient frequently exceeds a fifty percent dilution factor when testing with chemicals such as pesticides. The mechanical multichannel injection apparatus designed by Ozburn and Alasdair (1976) overcomes this problem, but its reproducibility and reliability depend heavily upon smooth operation of the mechanical components. For this reason the system is not recommended for use in chronic toxicity tests employing salt water as the diluent because excessive exposure to salt water may result in deterioration of the metal by corrosion (Ozburn, 1976).

The calibration of the toxicant delivery system should be checked carefully before, during, and after each test. This should include determining the volume of stock solution and dilution of water used in each portion of the toxicant delivery system and the flow-rate through each test container. The general operation of the toxicant delivery system should be checked daily during the test (U.S. EPA, 1975a).

- Dilution water

A minimal criterion for an acceptable dilution water is that healthy test organisms will survive in it for the duration of acclimation and testing without showing signs of stress such as discoloration or unusual behavior.

o Freshwater

Water in which Daphnids (which are more sensitive to many toxicants than most other freshwater animals) will survive and reproduce satisfactorily should be an acceptable water for most tests with freshwater animals.

o Estuarine and marine water

Water in which Acartia Tonsa or Mysid shrimp (which are more sensitive to many toxicants than most other estuarine and marine aquatic animals) will survive, grow, and reproduce satisfactorily should be an acceptable dilution water for most tests with estuarine and marine animals. If a dilution water is prepared from a dechlorinated water, it must be shown that in fresh samples of the dilution water taken daily during flow-through tests, the concentration of residual chlorine is less than 3μg per liter or that Acartia Tonsa, Mysid shrimp, oyster larvae or first instar Daphnids can survive for 48 hours without food.

- Reconstituted water

The recommended reconstituted waters (Table 3.3.1, 3.3.2 and 3.3.3) should be used as dilution water for as many tests as possible to maximize the number of reliable comparisons that can be made concerning relative toxicity and relative sensitivity. Reconstituted water is prepared by adding a known amount of specified reagent-grade chemicals to water which meets the specifications in Tables 3.3.1, 3.3.2, and 3.3.3.

- Alternative water

Alternative dilution water should be uncontaminated and of constant quality and should meet the following specifications:

Suspended solids	20 mg/l
TOC	10 mg/l
Un-ionized ammonia	20 µg/l
Residual chlorine	3 µg/l
Total organophosphorus pesticides	50 ng/l
Total organochlorine pesticides plus PCB's	50 ng/l

For effluent tests, the dilution water must be a representative sample of the receiving water obtained as close to the point of discharge as possible, but upstream of or outside the zone of influence of the effluent. For tests with freshwater organisms, municipal water supplies often contain unacceptable concentrations of copper, lead, zinc, fluoride, and chlorine or chloramine. Metals can be remonved by chelating resins. Sodium bisulfite is better for dechlorinating water than sodium sulfite, and both are much more reliable that a carbon filter, especially for removing chloramine (U.S. EPA, 1975a).

- Cleaning of test chambers, delivery systems, holding tanks, etc.

Toxicant delivery systems and test chambers must be cleaned before use. New ones must be washed with detergent and rinsed with fresh tap water. At the end of every test, if the toxicant delivery systems or test chambers are to be used again, they should be:

- emptied
- cleaned by a procedure appropriate for removing the toxicant tested (e.g., acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic compounds)
- rinsed twice with water

Acid is useful for removing mineral deposits, and 200 mg of hypochlorite per liter or 30% formalin plus 1% benzalkonium chloride are useful for removing organic matter and for disinfection. However, acid and hypochlorite must not be used together. Test chambers and toxicant delivery systems must be rinsed with dilution water just before use. Holding and acclimation tanks should be sterilized with an Iodophor or with 200 mg of hypochlorite per liter for 1 hour, scrubbed well once during the hour and rinsed will between groups of test organisms (U.S. EPA, 1975a).

TABLE 3.3.1 QUANTITIES OF REAGENT-GRADE CHEMICALS REQUIRED TO PREPARE RECOMMENDED RECONSTITUTED FRESH WATERS AND THE RESULTING WATER QUALITIES (Marking and Dawson, 1973)

Name	Salts Required (mg/l)				pH[a]	Hardness[b]	Alkalinity[b]
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl			
Very soft	12	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Hard	192	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384	240.0	240.0	16.0	8.0-8.4	280-320	225-245

[a] Approximate equilibrium pH after aeration and with fish in water.
[b] Expressed in mg/l as $CaCO_3$.

TABLE 3.3.2 QUANTITIES OF REAGENT-GRADE CHEMICALS TO BE ADDED TO AERATED SOFT RECONSTITUTED FRESH WATER FOR BUFFERING pH (Marking and Dawson, 1973) (The solutions should not be aerated after addition of these chemicals.)

pH[a]	Milliliters of Solution for 15 Liters of Water		
	1.0N NaOH	0.0M KH_2PO_4	0.5M H_3BO_3
6.0	1.3	80.0	---
6.5	5.0	30.0	---
7.0	19.0	30.0	---
7.5	---	---	---
8.0	19.0	20.0	---
8.5	6.5	---	40.0
9.0	8.8	---	30.0
9.5	11.0	---	20.0
10.0	16.0	---	18.0

[a] Approximate equilibrium pH with fish in water.

TABLE 3.3.3 RECOMMENDED PROCEDURE FOR PREPARING RECONSTITUTED SEA WATER (Kester et al., 1967; Zaroogian et al., 1969; Zillioux et al., 1973)

(Add the following reagent-grade chemicals in the amounts and order listed to 890 ml water. Each chemical must be dissolved before another is added.)

Chemical	Amount	Chemical	Amount
NaF	3 mg	Na_2SO_4	4.00 g
$SrCl_2 \cdot 6H_2O$	20 mg	$MgCl_2 \cdot 6H_2O$	10.78 g
H_3BO_3	30 mg	NaCl	23.50 g
KBr	100 mg	$Na_2SiO_3 \cdot 9H_2O$	20 mg
KCl	700 mg	Na_4EDTA	1 mg
$CaCl_2 \cdot 2H_2O$	1.47 g	NaHCO	200 mg

- Laboratory instrumentation calibration

All calibration of instruments used for water quality analyses must be documented on an appropriate laboratory data sheet. This is accomplished by recording the following information:

- Date
- True value of standards and calibration value
- Factor, if any, required to correct reading from meter
- Amount of drift
- Initials of person performing calibration

The following is a list of instruments that require calibration:

- Laboratory pH meter

Calibrate with two standard buffer solutions that cover the pH range of the samples being analyzed. Calibrate at start of testing (daily) and check for drift with one buffer solution periodically during laboratory use.

- Laboratory dissolved oxygen meter

Calibrate by running modified Winkler Full Bottle Technique (U.S. EPA, 1973) on three samples. Average and calibrate to this value. Run a final Winkler daily to check for drift upon completion of analysis.

- Temperature meter (Dissolved oxygen meter)

Calibrate with NBS thermometer semiannually.

- Conductivity meter

Standarize monthly with standard potassium chloride (0.01M) as stated in "Standard Methods", 14th Edition (Rand et al, 1975).

o Refractometer

Calibrate with water (U.S. EPA, 1975b).

3.3.1.3. Test substance--

The test substance can be one or more pure chemicals, a complex mixture such as formulation, or an effluent. Sometimes, the test solutions are not true solutions because they contain undissolved toxic agents.

o Basic test

The toxicant should be added to the dilution water or the toxicant delivery system without the use of any solvent or other additive, except water, if possible (U.S. EPA, 1975a).

If a carrier or vehicle is used to dissolve or dilute the test substance, it should possess as many of the following characteristics as possible:

- it should not interfere with absorption, distribution, metabolism, or retention of the test substance
- it should not alter the chemical properties of the test substance or enhance, reduce, or alter the toxic characteristic of the test substance
- it should not affect the food and water consumption of the test organism
- at the levels used in the study, it should not produce physiological effects or have local or systemic toxicity (Anon., 1977).

In addition, such a carrier or vehicle should, if possible, closely resemble the substance to be used under expected conditions of use (Anon., 1977).

The calculated concentrations of the additives to which any test organism are exposed must never exceed one twentieth of the concentration of the toxicant and must never exceed one-tenth gram per liter of water. Two sets of controls must be used, one exposed to no additive and one exposed to the highest level of additive to which any other organisms in the test are exposed (U.S. EPA, 1973).

The test substance should be of technical-grade. The lot and purity of the test substance should be known and recorded. The stability of the test substance in the stock solution should be determined. For long-term studies, when the test substance is incorporated into the dilution water, the concentration of the test substance in the dilution water should be determined at the start of the study and samples collected periodically to verify the concentration (Anon., 1977).

- Effluent Test

The test substance may be a sample of an effluent. Such a sample must not be aerated or altered in any way except that it may be filtered through a sieve or screen with 2-mm or larger holes. Samples must be covered at all times and violent agitation must be avoided. The collection of samples should be based on an understanding of the short-and long-term operations and schedules of the discharger if possible.

o For effluent static tests, separate tests generally should be conducted on at least two grab samples and more tests may often be desirable, especially if there are known sources of variability such as process changes. Tests should be begun as soon as possible, but must be begun within 8 hours, after the sample is obtained. The temperature of the sample should be adjusted to the test temperature ($\pm 2^{\circ}C$) and maintained at that temperature until portions are added to the dilution water. Often it is convenient to store the sample in the constant temperature water bath or area in which the test chambers are placed during the test.

o For effluent flow-through tests, the sample of the effluent must be taken continuously from the discharge line and introduced directly into a small effluent headbox that feeds the toxicant delivery system. If the discharge rate is not reasonably constant, flow-proportional continuous sampling may be desirable. For effluents that are only discharged in batches, a grab sample must be used and the test must begin within 8 hours after the sample is obtained. The temperature of the sample should be adjusted to be within the allowable test temperature range before it is added to the dilution water.

o Special effluent tests may be conducted on altered or treated samples of the effluent or on other samples to obtain additional information concerning the toxicity of the effluent. When special tests are conducted, the exact methodology must be described in all test reports (U.S. EPA, 1975a).

- Periodic Check of Concentration

During the test, it is desirable to measure the concentration of the test substance in the test chambers as often as practical. At a minimum, the concentration of the test substance must be measured in:

o each test chamber at least once during the test
o at least one test chamber at the next to the lowest test substance concentration at least once every 24 hours during the test
o at least one appropriate test chamber whenever malfunction is detected in any part of the toxicant delivery system

For replicate test chambers at the same test substance concentration, the highest measured concentration divided by the lowest measured concentration must be less than 1.2. If it is not, the toxicant delivery system should be checked and additional samples from the proper test chambers should be analyzed to determine if the sampling or analytical methods are precise enough. In addition, the measured concentration of the test substance in any test chamber must be no more than 30% higher or lower than the concentration calculated from the composition of the stock solution and the calibration of the toxicant delivery system. Measurement of degradation products of the test substance is desirable (U.S. EPA, 1975).

Whenever samples from a toxicity test are analyzed, at least one reagent blank must also be analyzed, if appropriate. Also, at least one sample for the method of known additions must be prepared by adding test substance to water from a control test chamber to match the next to the lowest test substance concentration used in the toxicity test. Methods used for analysis of test substances must be those specified in the latest edition of the Annual Book of Standards, Part 31 (American Society for Testing Materials, 1974) or methods for Chemical Analysis of Water and Wastes (U.S. EPA, 1974a). The accuracy of standard solutions should be checked against other standard solutions whenever possible. Atomic absorption spectrophotometric methods for metals and gas chromatographic methods for organic compounds are generally preferable to colorimetric methods (U.S. EPA, 1975a).

Reagent grade chemicals should be used in all tests. All reagents should conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination (ASTM, 1974b).

- Standard toxicant

To insure that the technical aspects of the bioassay are properly performed, an internal standard is recommended (LaRoche et al., 1970). The compound used routinely is sodium dodecyl sulfate (SDS), a surfactant and membrane lytic agent. This compound produces a very sharp response curve indicating an almost "all or none" effect at concentrations of 1 to 2 mg/l. While the use of an internal standard can serve as a quality assurance monitor, it does not, in itself, validate an experiment. Adequate control survival ($\geq$ 85%) is the primary criterion for the success or failure of a bioassay.

- Toxicant concentration selection

Generally a broad range of concentrations covering at least four orders of magnitude is chosen initially. This is followed by a progressive bisection of intervals on a logarithmic scale (Table 3.3.4) or decilog intervals (Table 3.3.5) (Rand et al., 1975).

TABLE 3.3.4 GUIDE TO SELECTION OF EXPERIMENTAL CONCENTRATIONS, BASED ON PROGRESSIVE BISECTION OF INTERVALS ON LOGARITHMIC SCALE (Rand et al., 1975)

Column 1	Column 2	Column 3	Column 4	Column 5
10.0	---	---	---	8.7
---	---	---	7.5	---
---	---	---	---	6.5
---	---	5.6	---	---
---	---	---	---	4.9
---	---	---	4.2	---
---	---	---	---	3.7
---	3.2	---	---	---
---	---	---	---	2.8
---	---	---	2.4	---
---	---	---	---	2.1
---	---	1.8	---	---
---	---	---	---	1.55
---	---	---	1.35	---
---	---	---	---	1.15
1.0	---	---	---	---

TABLE 3.3.5 GUIDE TO SELECTION OF EXPERIMENTAL CONCENTRATIONS, BASED ON DECILOG INTERVALS (Rand et al., 1975)

Concentrations		Log of Concentration
Column 1	Column 2	
10.0		1.00
	7.94 (or 7.9)	0.90
6.31 (or 6.3)		0.80
	5.01 (or 5.0)	0.70
3.98 (or 4.0)		0.60
	3.16 (or 3.15)	0.50
2.51 (or 2.5)		0.40
	1.99 (or 2.0)	0.30
1.58 (or 1.6)		0.20
	1.26 (or 1.25)	0.10
1.00		0.00

- Sample collection and handling

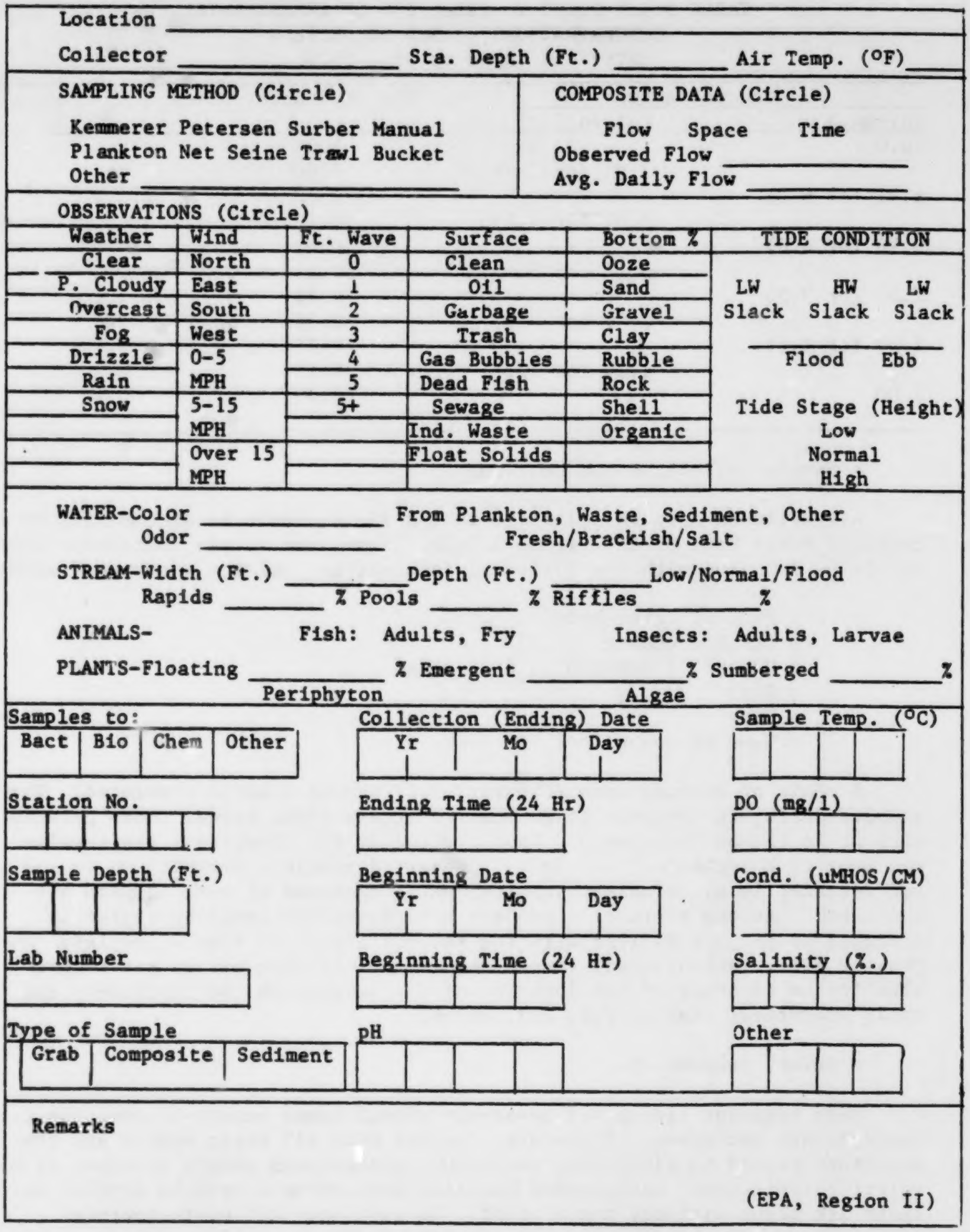
All effluent samples collected in the field should be accompanied by a complete Field Data Sheet (Figure 3.3.1). Also, the sample containers used should be labelled with the following information, using a waterproof marker:

- o Name of water body
- o Station number
- o Number of subsamples of sample
- o Date
- o Time
- o Name of collector.

A chain of custody form (Figure 3.3.2) should also be completed. The samples during the transit stage must be at all times either under personal care or in locked containers. Upon arrival at the laboratory the samples are kept in a locked cabinet (e.g., preserved sample - benthic) or locked refrigerator (e.g., bioassay samples) until analyses of such samples are initiated. At the start of a project a professional level biologist is assigned as project officer with the responsibility to keep a complete project file, including all record sheets. It is also his or her responsibility to be aware of the location of the samples in the laboratory and their analytical status (U.S. EPA, 1975b).

- Safety precautions

Many toxicant agents can adversely affect human beings if adequate precautions are not taken. Therefore, contact with all toxic agents and test solutions should be minimized, and special precautions should be taken with volatile toxicants. Recommended handling procedures should be studied before tests are begun with any toxic agent. Because many effluents contain sanitary wastes, the investigators should be inoculated for typhoid, polio, and tetanus before effluent tests are begun.



Location ____________________

Collector ____________ Sta. Depth (Ft.) ________ Air Temp. (°F)____

SAMPLING METHOD (Circle)

Kemmerer Petersen Surber Manual
Plankton Net Seine Trawl Bucket
Other ____________________

COMPOSITE DATA (Circle)

Flow Space Time
Observed Flow ____________
Avg. Daily Flow ____________

OBSERVATIONS (Circle)

Weather	Wind	Ft. Wave	Surface	Bottom %	TIDE CONDITION
Clear	North	0	Clean	Ooze	
P. Cloudy	East	1	Oil	Sand	LW HW LW
Overcast	South	2	Garbage	Gravel	Slack Slack Slack
Fog	West	3	Trash	Clay	
Drizzle	0-5	4	Gas Bubbles	Rubble	Flood Ebb
Rain	MPH	5	Dead Fish	Rock	
Snow	5-15	5+	Sewage	Shell	Tide Stage (Height)
	MPH		Ind. Waste	Organic	Low
	Over 15		Float Solids		Normal
	MPH				High

WATER-Color ____________ From Plankton, Waste, Sediment, Other
Odor ____________ Fresh/Brackish/Salt

STREAM-Width (Ft.) ________Depth (Ft.) ________Low/Normal/Flood
Rapids ________ % Pools ________ % Riffles________%

ANIMALS- Fish: Adults, Fry Insects: Adults, Larvae

PLANTS-Floating ________ % Emergent ________% Sumberged ________%
Periphyton Algae

Samples to:

Bact	Bio	Chem	Other

Collection (Ending) Date

Yr	Mo	Day

Sample Temp. (°C)

Station No.

Ending Time (24 Hr)

DO (mg/l)

Sample Depth (Ft.)

Beginning Date

Yr	Mo	Day

Cond. (uMHOS/CM)

Lab Number

Beginning Time (24 Hr)

Salinity (%.)

Type of Sample

Grab	Composite	Sediment

pH

Other

Remarks

(EPA, Region II)

Figure 3.3.1 Field data sheet. (U.S. EPA, 1975b)

<table>
<tr><td colspan="8">Name of Unit and Address:</td></tr>
<tr><td>Number</td><td>Unit</td><td colspan="6">Description of Samples</td></tr>
<tr><td></td><td></td><td colspan="6"></td></tr>
<tr><td colspan="6">Person Assuming Responsibility for Samples:</td><td>Time</td><td>Date</td></tr>
<tr><td>Number</td><td>Relinquished By:</td><td>Time</td><td>Date</td><td>Received By:</td><td>Time</td><td>Date</td><td>Reason for Change of Custody</td></tr>
<tr><td>Number</td><td>Relinquished By:</td><td>Time</td><td>Date</td><td>Received By:</td><td>Time</td><td>Date</td><td>Reason for Change of Custody</td></tr>
<tr><td>Number</td><td>Relinquished By:</td><td>Time</td><td>Date</td><td>Received By:</td><td>Time</td><td>Date</td><td>Reason for Change of Custody</td></tr>
</table>

(EPA, REGION II)

Figure 3.3.2 Chain of custody form (EPA, Region II)

Although disposal of test solutions and test organisms poses no special problem in most cases, health and safety precautions should be considered before the beginning of a test.

Rinsing with acetone and other volatile solvents should be performed only in well-ventilated areas.

3.3.1.4 Test subject--

An organism suitable as a test subject for Aquatic Bioassay must possess a number of characteristics (Rand et al., 1975):

- Sensitivity to the material or environmental factors under consideration
- Wide geographical distribution, abundance and availability throughout the year to allow comparative studies of control and exposed organisms under different environmental conditions and different locations
- Availability of culture methods for its rearing in the laboratory and knowledge of its environmental requirements
- Known recreational, economic, and ecological importance locally and nationally
- Good general physical condition and freedom from parasites and diseases.

The susceptibility of the test organisms to particular test substances is an important factor to consider prior to choosing the test species. Ideally, the most sensitive resident species should be bioassayed. Then, the distribution of the test organism within the system being assayed should be considered, because ideally the organisms selected should be among the representative species of the natural population (Martin, 1973).

The following sections discuss the species most sensitive to selected chemicals and having other desirable characteristics as test subjects.

- Fish, Macroinvertebrates, and Amphibians

For acute toxicity studies, the following species have been found suitable as test organisms, because they are extremely sensitive to the test chemicals (See Table 3.3.6), they have wide geographic distribution, abundance, and availability throughout the year, and they adapt easily to laboratory conditions:

TABLE 3.3.6 24-, 40-, and 96-HOUR LC50 VALUES FOR THE SPECIES OF FRESHWATER AND ESTUARINE ORGANISMS MOST SENSITIVE TO SELECTED CHEMICALS

Chemicals	24-Hour (mg/l)		40-Hour (mg/l)		96-Hour (mg/l)	
	Most Sensitive Species	LC50	Most Sensitive Species	LC50	Most Sensitive Species	LC50
ALDRIN	Oncorhynchus tshawytscha (chinook salmon)	0.0124	Oncorhynchus tshawytscha (chinook salmon)	0.0106	Morone saxatilis (striped bass)	0.0072
AROCHLOR 1016	---	---	--	---	Crassostrea virginica (oyster)	0.102
B.H.C.	Lepomis macrochirus (bluegill)	10.0	Lepomis macrochirus (bluegill)	7.8	Panaeus duorarun (pink shrimp)	0.00034
CHLORDANE	Lepomis macrochirus (bluegill)	0.036	Lepomis macrochirus (bluegill)	0.032	Panaeus duorarun (pink shrimp)	0.0004
DDT	Papaemonetes sp. (grass shrimp)	0.0007	Salmo clarki (cutthroat trout)	0.0016	Panaeus aztecus (brown shrimp)	0.0001
DURSBAN	Palaemonetes sp. (grass shrimp)	0.0032	---	---	---	---
DIELDRIN	Lepomis macrochirus (bluegill)	0.014	---	---	Panaeus duorarun (pink shrimp)	0.0007
ENDOSULFAN	Lepomis macrochirus (bluegill)	0.0036	Panaeus aztecus (brown shrimp)	0.0004	Panaeus duorarum (pink shrimp)	0.00004
ENDRIN	Salmo gairdneri (rainbow trout)	0.00079	Fundulus similis (longnose killifish)	0.00023	Panaeus duorarun (pink shrimp)	0.0001
HEPTACHLOR 65%	---	---	---	---	Panaeus duorarun (pink shrimp)	0.0001
HEPTACHLOR 74%	Pimephales promelas (fathead minnow)	0.013	Oncorhynchus tshawytscha (chinook salmon)	0.026	Panaeus duorarun (pink shrimp)	0.0001
HEPTACHLOR 99%	---	---	---	---	Panaeus duorarun (pink shrimp)	0.00003
LINDANE	Gasterosteus aculeatus (stickleback)	0.50	Pimephales promelas (fathead minnow)	0.070	Panaeus duorarun (pink shrimp)	0.0002
MALATHION	Palaemonetes sp. (grass shrimp)	0.032	Panaeus duorarum (pink shrimp)	0.0125	Panaeus duorarun (pink shrimp)	0.0125
METHOXYCHLOR	---	---	---	---	Panaeus duorarun (pink shrimp)	0.0035
PARATHION	Poecilia reticulata (guppy)	82.0	Poecilia reticulata (guppy)	68.0	Poecilia reticulata (guppy)	56.0
TOXAPHENE	Lepomis macrochirus (bluegill)	0.006	Gambusia affinis (mosquito fish)	0.024	Cyprinodon variegatus (sheepshead minnow)	0.0011
2,4-D	Gambusia affinis (mosquito fish)	7.0	Lepomis macrochirus (bluegill)	1.1	---	---
CARBARYL	Oncorhynchus kisutch (coho salmon)	0.003	Garassius auratus (goldfish)	0.11	Oncorhynchus kisutch (coho salmon)	0.0013
MOLINATE	Palaemonetes sp. (grass shrimp)	22.0	Palaemonetes sp. (grass shrimp)	20.0	Palaemonetes sp. (grass shrimp)	16.0
PROPANIL	Gambusia affinis (mosquito fish)	11.3	Gambusia affinis (mosquito fish)	11.0	Gambusia affinis (mosquito fish)	9.46

(continued)

TABLE 3.3.6 (Continued)

Chemicals	24-Hour (mg/l) Most Sensitive Species	24-Hour (mg/l) LC50	40-Hour (mg/l) Most Sensitive Species	40-Hour (mg/l) LC50	96-Hour (mg/l) Most Sensitive Species	96-Hour (mg/l) LC50
TRIFLURALIN	---	---	*Lepomis macrochirus* (bluegill)	0.019	---	---
KEPONE	---	---	---	---	*Leiostomus xanthurus* (spot)	0.0066
L.A.S.	*Pimephales promelas* (fathead minnow)	1.9	*Pimephales promelas* (fathead minnow)	1.7	---	---
PHENOL	---	---	*Lepomis macrochirus* (bluegill)	20.5	*Lepomis macrochirus* (bluegill)	19.3
CADMIUM	*Crangon septemspinosa* (sand shrimp)	2.4	*Crangon septemspinosa* (sand shrimp)	0.50	*Salmo gairdneri* (rainbow trout)	0.0010
COPPER	*Pimephales promelas* (fathead minnow)	0.04	*Pimephales promelas* (fathead minnow)	0.023	*Pimephales promelas* (fathead minnow)	0.022
CHROMIUM	*Pimephales promelas* (fathead minnow)	39.6	*Pimephales promelas* (fathead minnow)	19.7	*Pimephales promelas* (fathead minnow)	17.6
LEAD	*Pimephales promelas* (fathead minnow)	8.18	*Pimephales promelas* (fathead minnow)	5.9	*Salmo gairdneri* (rainbow trout)	1.17
MERCURY	*Morone saxatilis* (striped bass)	0.22	*Morone saxatilis* (striped bass)	0.14	*Morone saxatilis* (striped bass)	0.09
NICKEL	*Morone saxatilis* (striped bass)	10.0	*Poecilia reticulata* (guppy)	6.7	*Poecilia reticulata* (guppy)	4.45
ZINC	*Morone saxatilis* (striped bass)	11.2	*Cyprinus carpio* (carp)	9.3	*Salmo gairdneri* (rainbow trout)	0.430

- Grass Shrimp - *Palaemonetes sp*.
- Pink Shrimp - *Panaeus duorarum*
- Brown Shrimp - *Panaeus aztecus*
- Sand Shrimp - *Crangon septemspinosa*
- Fathead Minnow - *Pimephales promelas*
- Sheepshead Minnow - *Cyprinodon variegatus*
- Rainbow Trout - *Salmo gairdneri*
- Cutthroat Trout - *Salmo clarki*
- Coho Salmon - *Oncorhynchus kisutch*
- Chinook Salmon - *Oncorhynchus tshawytscha*
- Bluegill Sunfish - *Lepomis macrochirus*
- Stickleback - *Gasterosteus aculeatus*
- Killifish - *Fundulus similis*
- Mosquito Fish - *Gambusia affinis*
- Guppy - *Poecilia reticulata*
- Striped Bass - *Morone saxatilis*
- Gold Fish - *Carassius auratus*
- Carp - *Cyprinus carpio*
- Spot - *Leiostomus xanthurus*

- Macroinvertebrates

Daphnia magna was found to be the most senitive animal to herbicides (Table 3.3.7) followed in descending order of sensitivity by seed shrimp, scud, glass shrimp, sowbug and crayfish (Sanders, 1970). In a study of acute toxicity of various metals to freshwater zooplankton (Table 3.3.8), *Daphnia hyalina* was more sensitive than either *Cyclop abyssorum* and *Eudiaptomus padanus*. The high sensitivity of *Daphnia* makes this invertebrate a useful test organism for heavy metal pollutants.

In addition, *Daphnia* fulfills a whole series of requirements for an animal to be used in water pollution tests:

- it is easy to find everywhere
- it is of small size but not miscroscopic
- it has a simple level of organization thus avoiding secondary effects of toxic chemicals
- it is of rapid reproduction and easy to breed in the laboratory (Baudouin and Scoppa, 1974).

- Aquatic Insects

Aquatic insects to be used as biological monitors of heavy metal fishkills must fulfill three prerequisites (Nehring, 1976):

- The insect should be more tolerant of the heavy metals than the fish in question

TABLE 3.3.7 THE 48-HR TL50 (mg/1) OF SOME HERBICIDES TO SIX SPECIES OF FRESHWATER CRUSTACEANS AT TWO DIFFERENT TEMPERATURES (SANDERS, 1970)

HERBICIDE	Waterflea Daphnia magna 21°C	Seed Shrimp Cypridopsis vidua 21°C	Scud Gammarus fasciatus 15.5°C	Sowbug Asellus brevicaudus 15.5°C	Glass Shrimp Palaemonetes kadiakensis 21°C	Crayfish Orconectes Snails 15.5°C
Diclone	0.025	0.12	0.24	0.20	0.45	3.2
2,4.D	0.10	0.32	2.6	2.2	2.7	100.0
Silvex (P.G. BE)	0.18	0.20	1.0	0.50	3.2	100.0
Trifuralin	0.56	0.25	1.8	2.0	1.2	50.0
Molinate	0.60	0.18	0.39	0.40	1.0	5.6
Simazine	1.0	3.2	100.0	100.0	100.0	100.0
Vernolate	1.1	0.24	20.0	5.6	1.9	24.0
Silvex (B.E.E)	2.1	4.9	0.74	40.0	8.0	60.0
2,4.D (Dimethylamine salt)	4.0	8.0	100.0	100.0	100.0	100.0
2,4.D (B.E.E.)	5.6	1.8	5.9	3.2	1.4	100.0
Dichlobenil	10.0	7.8	18.0	34.0	9.0	22.0
Amitrol - T	30.0	32.0	100.0	100.0	100.0	100.0
Diphenamid	56.0	50.0	100.0	100.0	58.0	100.0

TABLE 3.3.8 ACUTE TOXICITY OF VARIOUS METALS (mg/l, 48 hour TL50) TO FRESH WATER ZOOPLANKTON (Baudouin and Scoppa, 1974)

Metal	*Cyclops abyssorum*	*Eudiaptomus padanus*	*Daphnia hyalina*
Calcium	7000	4000	3000.0
Magnesium	280.0	180.0	32.0
Strontium	300.0	180.0	75.0
Cesium	400.0	135.0	7.4
Chromium VI	10.0	10.1	0.022
Cobalt	15.5	4.0	1.32
Nickel	15.0	3.6	1.90
Lead	5.5	4.0	0.60
Mercury	2.2	0.85	0.0055
Zinc	5.5	0.50	0.040
Cadmium	3.8	0.55	0.055
Copper	2.5	0.50	0.005

- The insects must concentrate the toxic metal in relative proportion to the metal content of the water

- The insects must concentrate the metal pollutant by some predictable factor over a short time period

In this kind of experimentation a good bio-accumulator is desirable.

Toxicity data for three aquatic insects are given in Table 3.3.9 (Warnick and Bell,1969). A comparison of the TL50 values of lead, zinc, copper, nickel and cadmium to toxicity in fish, i.e., stickleback (TL50 mg/l for Zn = 0.01-10.0; for Cu = 0.01-0.02; for Ni = 0.08-1.0; for Pb = 0.1-0.4; for Cd = 0.03), reveals aquatic insects to be more tolerant of all heavy metals tested. The Mayfly, however, was less tolerant of silver than rainbow trout (Jones, 1938). Tables 3.3.10 - 3.3.13 (Nehring, 1976) compare the levels of accumulation in the insect with the levels of exposure. In each test, the average level of exposure was paired with the corresponding average accumulation level in the insect. The correlation coefficients in seven of the fourteen bioassays were 0.97 or greater (Table 3.3.14). These correlation coefficients indicate that aquatic insects accumulate heavy metals in relative proportion to the metal concentration in the water.

TABLE 3.3.9 THE ACUTE TOXICITY OF SOME HEAVY METALS TO AQUATIC INSECTS (Warnick and Bell, 1969)

Metal	Insect	96-hr TL50 (mg/l)	50% Survival (days)	(mg/l)
Cu^{++} from $CuSO_4 \cdot 5H_2O$	acroneuria	8.3 (0.32 48-hr)		
	ephemerella			
	hydropsyche		14	32.0
Zn^{++} from $ZnSO_4 \cdot 7H_2O$	acroneuria		14	32.0
	ephemerella		10	16.0
	hydropsyche		11	32.0
Cd^{++} from $CdSO_4 \cdot 8H_2O$	acroneuria		14	32.0
	ephemerella	2.0		
	hydropsyche			
Pb^{++} from $PbSO_4$	acroneuria		14	64.0
	ephemerella		7	16.0
	hydropsyche		7	32.0
Fe^{++} from $FeSO_4$	acroneuria		> 14	64.0
	ephemerella	0.32	7	16.0
	hydropsyche		7	32.0
Ni^{++} from $NiSO_4 \cdot 6H_2O$	acroneuria	33.5		
	ephemerella	4.0		
	hydropsyche		> 14	64.0
Co^{++} from $CoSO_4 \cdot 7H_2O$	acroneuria		8	32.0
	ephemerella	16.0		
	hydropsyche		7	32.0

TABLE 3.3.10 COPPER BIOASSAYS, AVERAGE EXPOSURE vs. AVERAGE ACCUMULATION (Nehring, 1976)

Mayfly (2 Replications)		Stonefly (3 Replications)	
Exposure (mg/1)	Accumulation (µg/g)	Exposure (mg/1)	Accumulation (µg/g)
10.0	9,125	12.2	2,540
4.82	5,787	10.4	2,096
2.51	3,882	8.13	1,767
1.22	1,933	6.47	1,199
0.63	1,240	----	-----
0.00	94.7	0.00	122.3

TABLE 3.3.11 LEAD BIOASSAYS, EXPOSURE vs. ACCUMULATION (Nehring, 1976)

Mayfly		Stonefly	
Exposure (mg/1)	Accumulation (µg/g)	Exposure (mg/1)	Accumulation (µg/g)
9.24	104,700	19.2	8,172
4.90	73,200	7.44	2,249
2.34	31,780	4.43	1,666
1.32	14,560	1.96	736.6
0.69	5,702	1.08	716.7
0.00	126.6	0.00	8.18

TABLE 3.3.12 SILVER BIOASSAYS, AVERAGE EXPOSURE vs. AVERAGE ACCUMULATION (Nehring, 1976)

Mayfly (2 Replications)		Stonefly (3 Replications)	
Exposure (mg/1)	Accumulation (µg/g)	Exposure (mg/1)	Accumulation (µg/g)
0.75	65.31	0.738	53.28
0.40	36.65	0.399	30.76
0.23	47.97	0.217	22.95
0.12	28.73	0.105	13.62
0.06	25.32	0.050	9.13
0.00	0.00	0.000	3.97

TABLE 3.3.13 ZINC BIOASSAY, EXPOSURE vs. ACCUMULATION (Nehring 1976)

Mayfly		Stonefly	
Exposure (mg/1)	Accumulation (μg/g)	Exposure (mg/1)	Accumulation (μg/g)
9.20	2,361	13.6	561.2
4.32	2,381	5.54	497.1
2.29	2,187	2.83	415.7
1.04	2,029	1.61	507.7
0.60	1,794	0.77	439.4
0.00	1,116	0.00	357.2

TABLE 3.3.14 BIOASSAY PARAMETERS AND CORRELATION COEFFICIENTS (Nehring, 1976)

Test Metal	Test Insect	Range of Exposure (metal in mg/1)	Correlation Coefficient
Copper	Stonefly	0.74 - 13.9	0.986
Copper	Stonefly	5.51 - 18.5	0.901
Copper	Stonefly	6.47 - 12.2	0.994
Copper	Mayfly	0.63 - 10.0	0.982
Copper	Mayfly	0.08 - 1.06	0.974
Lead	Stonefly	1.08 - 19.2	0.991
Lead	Mayfly	0.69 - 9.24	0.985
Silver	Stonefly	0.05 - 0.74	0.996
Silver	Stonefly	0.004- 0.067	0.909
Silver	Stonefly	0.006- 0.104	0.830
Silver	Mayfly	0.06 - 0.75	0.893
Silver	Mayfly	0.01 - 0.15	0.666
Zinc	Stonefly	0.77 - 13.6	0.779
Zinc	Mayfly	0.60 - 9.20	0.694

The predictable factor, termed "concentration factor", is determined by dividing the average level of exposure into the average level of metal accumulation in the insect. The concentration factor is very effective in estimating the average level of exposure to lead, copper, and silver (Table 3.3.15) (Nehring, 1976). In 19 of 28 instances, the concentration factor estimated the actual level of exposure with an accuracy of 80% or better. In 10 of 28 instances, the concentration factor estimated the actual level of exposure with an accuracy of 90% or greater. Thus aquatic insects as tested do concentrate heavy metals by some predictable factor.

TABLE 3.3.15 EFFECTIVENESS OF CONCENTRATION FACTORS IN ESTIMATION OF AVERAGE LEVELS OF EXPOSURE TO LEAD, COPPER AND SILVER (Nehring, 1976)

Percent Accuracy	Frequency
50 - 59%	1/28
60 - 69%	3/28
70 - 79%	5/28
80 - 89%	9/28
90 - 99%	10/28

In summary, aquatic insects fulfill the three prerequisites mentioned on pages 231 and 233, and appear to be excellent biological monitors of heavy metal pollution. They are more tolerant of metal than fish, they accumulate metal in relative proportion to the metal concentration in the water and they concentrate the metal by some predictable factor.

- Benthos

o In a study (Hansen et al., 1974a) the American oyster, brown shrimp and grass shrimp were found to be about equally sensitive to Aroclor 1016 (Table 3.3.16).

TABLE 3.3.16 AROCLOR 1016 (Hansen et al., 1974a)

Test Organism	Scientific Name	96-hr. LC_{50} (mg/l)
Oyster	Crassostrea virginica	10.2
Brown Shrimp	Panaeus aztecus	10.5
Grass Shrimp	Palaemonetes sp.	12.5

In addition to its sensitivity, the American Oyster possesses a wide geographic range extending from Price Edward, Canada, along the Atlantic Coast to the Gulf Coast of Texas. It is now feasible to spawn adult oysters, rear the larvae, and maintain the spat and juvenile oysters under controlled laboratory conditions.

o The midge (Chironomus species) was found to be the most sensitive test organism to certain metals (Table 3.3.17) (Rehwoldt, et al., 1973):

Mercury++(24-hr LC50 = 0.06 mg/l)

Copper++(24-hr and 96-hr LC50 = 0.65 and 0.03 mg/l)

Nickel++(24-hr LC50 = 10.2 mg/l; 96-hr LC50 = 8.6 mg/l)

In the same study, the scud (Gammarus species) was the most sensitive organism to zinc++, cadmium++ and chromium++ in both 24 hour and 96 hour acute toxicity study, and to mercury++ in 96 hour.

o Green Algae (Dunaliella tertiolecta Butcher), found in marine and estuarine waters, has shown the most linear response for every parameter examined (McLachlan, 1960). An additional advantage of green algae is that it requires no outside sources of vitamins (Provasoli, 1963). Dunaliella tertiolecta has been shown to be a highly versatile and consistent bioassay organism for nutrient assessment in marine, estuarine, and some freshwater situations. It will respond to concentration at least as low as 2.5 mg phosphorus (P)/l; 10 mg ammonia (N)/l and 50 mg nitrate (N)/l in defined media (Specht and Miller, 1973). Green algae was also found to be one of the most sensitive species to herbicides (Table 3.3.18) (Hollister and Walsh, 1973).

The following are the average EC50 values (ppb) from Table 3.3.18 for four herbicides and four families of marine unicellular algae.

Family	Number of Species Tested	Neburon EC50	Diuron EC50	Atrazine EC50	Ametryne EC50
Chlorophyceae	6	23	22	104	31
Bacillariophyceae	8	77	67	265	65
Chrysophyceae	3	24	13	92	11
Phodophyceae	1	24	24	79	35

The family of Chrysophyceae as a whole was generally the most sensitive. In addition to Dunaliella tertiolecta, Skeletonema costatum is an ecologically important phytoplankton that is common to a wide geographic range of neritic waters and Thalassiosira pseudonana is sensitive to heavy metals and has an 8 hour generation time which offers great practical value in the establishment of toxicological responses. Both Skeletonema costatum and Thalassiosira pseudonana have been recommended by EPA (US. EPA, 1976).

TABLE 3.3.17 THE TOXICITY (LC50, mg/1) OF SOME HEAVY METAL IONS TOWARD BENTHIC ORGANISMS (Rehwoldt et al., 1973)

Test	Cu^{++}		Zn^{++}		Ni^{++}		Cd^{++}		Hg^{++}		Cr^{++}	
Organisms	24hr	96hr	24hr	96hr	24 hr	96hr	24hr	96hr	24hr	96hr	24hr	96hr
Bristle Worm	2.3	0.09	21.2	18.4	16.2	14.1	4.6	1.7	1.9	1.0	12.1	9.3
Scud (amphipoda)	1.2	0.91	10.2*	8.1*	15.2	13.0	0.14*	0.07*	0.09	0.01*	6.4*	3.2*
Caddis Fly	12.1	6.2	62.6	58.1	48.4	30.2	5.1	3.4	5.6	1.2	58	50
Damsel Fly (zygoptera)	10.2	4.6	32	26.2	26.4	21.2	11.0	8.1	3.2	1.2	46	43.1
Midge (Diptera)	0.65*	0.03*	21.5	18.2	10.2*	8.6*	5.1	1.2	0.06*	0.02	16.5	11.0
Snail (egg) (Gastropoda)	4.5	9.3	28.1	20.2	26.0	11.4	5.1	3.8	6.3	2.1	15.2	12.4
Snail (adult)	1.5	0.9	16.8	14.0	21.2	14.3	10.1	8.4	1.1	0.08	10.2	8.4

*Most sensitive

TABLE 3.3.18 EC50 (ppb) OF NEBURON, DIURON, ATRAZINE, AND AMETRYNE ON OXYGEN EVALUATION BY MARINE UNICELLULAR ALGAE. STANDARD ERRORS (SE) WERE DERIVED BY UNWEIGHTED PROBIT ANALYSIS (Hollister and Walsh, 1973)

Family Species	Neburon		Diuron		Atrazine		Ametryne	
	EC50	SE	EC50	SE	EC50	SE	EC50	SE
Chlorophyceae								
Chlamydomonas sp.	37	5	37	3	60	8	41	5
Dunaliella tertiolecta	10	3	10	3	159	18	40	6
Platymonas sp.	12	5	17	3	102	8	24	4
Chlorella sp.	22	3	19	2	143	8	32	3
Neochloris sp.	39	6	28	5	82	7	36	7
Chlorococcum sp.	20	3	20	4	80	7	10	3
Bacillariophyceae								
Thalassiosira fluviatilis	108	9	95	10	110	19	58	7
Navicula inserta	124	11	93	12	460	15	97	9
Amphora exigua	82	5	31	4	300	21	26	4
Achnanthes brevipes	23	4	24	1	93	11	19	1
Stauroneis amphoroides	17	3	31	2	348	67	65	11
Cyclotella nana	11	4	39	7	84	19	55	8
Nitzschia closterium	120	13	50	6	287	68	62	6
Nitzschia (Ind. 684)	131	9	169	17	434	84	135	11
Chrysophyceae								
Monochrysis lutheri	12	4	18	3	77	23	14	4
Isochrysis galbana	20	5	10	3	100	17	10	4
Phaeodactylum tricornutum	40	7	10	3	100	19	10	5
Rhodophyceae								
Porphyridium cruentum	24	4	24	3	79	9	35	3

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o The Diatom has been chosen frequently as bioassay organism because of the large number of species of Diatom present in almost all natural waters. These species have very different ranges of tolerance to ecological conditions. They are a very important food source for most forms of aquatic life that feed upon plants and they carry out the process of photosynthesis which is so important in the generation of oxygen needed by all organisms. Because they consist of many species that have populations composed of varying number of specimens, they are an excellent group to treat statistically in analyzing their reactions to varying ecological conditions. Furthermore, one can collect diatoms and retain them for long periods before study without losing their characteristics for identification (Glass, 1973).

In a study of the effect of copper upon the growth of phytoplankton, Cyclotella nana, a representative diatom, was found to possess a great sensitivity to copper (Table 3.3.19) (Erickson et al., 1970).

TABLE 3.3.19 THE GROWTH SENSITIVITY OF ALGAE TO COPPER
(Erickson et al., 1970)

Organism	µg Cu/l							
	0	50	100	500	100	1500	2000	3000
Porphyridium cruentum	+	+	+					
Monochrysis lutheri	+	+	+					
Nannochloris oculata	+	+	+					
Amphidinium carteri	+	+						
Skeletonema costatum	+	+	+					
Ohisthodiscus luteus	+	+						
Chaetoceros sp.	+							
Nitzschia closterium	+	+	+					
Platymonas subcordiformis	+	+	+					
Cyclotella nana	+							
Dunaliella tertiolecta	+	+	+	+	+	+	+	+
Isochrysis galbana	+	+	+					

+ = Visible growth after 14 days

Naricula seminulum, another species of diatom, was found to be the most sensitive to nitrilotriacetic acid (NTA) (Table 3.3.20) (Sturm and Payne, 1973). An additional advantage of diatoms is that the use of unialgal diatom cultures for laboratory bioassay analysis has been an accepted ASTM (American Society for Testings and Materials) method for several years (Patrick, 1964).

TABLE 3.3.20 THE COMPARATIVE STATIC, ACUTE TOXICITY OF NTA TO
BLUEGILLS, SNAILS, AND DIATOMS EXPRESSED AS mg/l
(Sturm and Payne, 1973)

Test Organisms	Scientific Name	96 hr TL50 mg/l	Water Hardness mg/l CaCo$_3$
Bluegill	Lepomis macrochirus	252	60
Snails	Physa leterastropha	373	60
Diatoms	Naricula seminulum	185	60
Bluegill	Lepomis macrochirus	487	170
Snails	Physa leterastropha	522	170
Diatoms	Naricula seminulum	477	170

In addition to the diatom, the following species have been successfully utilized in bioassay and have been proposed by EPA in the algal assay Bottle test (Payne, 1975).

- *Selenastrum capricornutum*
- *Microcystis aeruginosa*
- *Anabaena flos-aquae*

Of the three species selected, Selenastrum capricornutum is the easiest to culture and to use in testing. Its growth rate is approximately twice that of the two blue-green Microcystis aeruginosa and Anabaena flos-aquae. Its growth responses normally are more clearly nutrient dependent and test results, therefore, are easier to interpret.

- Protozoans

Protozoa, algae, and bacteria form the broad bases of the aquatic food chain. Ciliates are among the most numerous organisms of the estuarine benthos (Borror, 1963), and may be most important as nutrient regenerators (Johannes, 1965). Also, some ciliates, including Tetrahymena pyriformis, can concentrate certain pesticides and PCB's (Cooley et al., 1972; Gregory et al., 1969). Tetrahymena pyriformis has been used as test organism (Rand et al., 1975) for the following reasons:

- it occurs in freshwater and salt marshes
- it has world-wide distribution
- it is readily grown in axenic culture
- its physiology has been studied extensively

Tetrahymena pyriformis strain W and HSM. has been used successfully in many bioassays (Elliott et al., 1973; Corliss, 1970).

The sensitivity of T. pyriformis strain W to insecticide is shown in Table 3.3.21 (Cooley, 1973).

TABLE 3.3.21 SENSITIVITY OF T. PYRIFORMIS, STRAIN W, TO INSECTICIDES (Cooley, 1973)

Toxicant	Growth Rate reduction	96-hr. population density reduction	Accumulation (X initial concentration)
Mirex	33% at 0.9 μg/l	12% at 0.9 μg/l	193 X
Aroclor 1248	18.9% at 1 mg/l	9.6% at 1 mg/l	48 X
Aroclor 1254	8% at 1 μg/l	10% at 1 μg/l	60 X
Aroclor 1260	19.1 to 25% at 1 mg/l	13.6 to 22.4% at 1 mg/l	79 X

These data indicate that a significant reduction in population growth and 96-hr population density occurred at low toxicant concentrations. T. pyriformis, strain HSM, has been chosen as test species because:

- it is a large, mobile cell, easy to observe and count under relatively low power of magnification
- it has been in culture for 30 years without known genetic change
- its cell is easily grown and has a generation time of 3 hrs at room temperature.

Table 3.3.24 shows the lethal concentrations of certain heavy metals for Tetrahymena and several species of fish.

These data suggest that with the exception of lead nitrate, T. pyriformis is a more sensitive indicator of water pollution due to heavy metal contamination than fish. In addition, T. pyriformis is, in turn, part of the zooplankton which serves as food for organisms higher in the food chain. Therefore, toxic damage to T. pyriformis should give an indication that harmful changes are likely to occur in those organisms which are higher in the food chain (Carter and Cameron, 1973).

- Microorganisms

Keil et al. (1972) described a commercial PCB formulation at a concentration of 0.1 μg/ml which stimulated the growth of Escherichia coli. Little information on the interactions of PCB's with heterotrophic microorganisms is available (Keil et al., 1972). Bourquin et al. (1975), in the study of inhibition of growth of estuarine bacteria by PCB, came to the realization that most of the sensitive bacteria were gram-positive (Table 3.3.23).

In addition, Trudgill et al. (1971) performed a test on the comparative effects of organochlorine on bacterial growth (Table 3.3.24). The gram-positive bacteria were found to be more sensitive than gram-negative bacteria and, particularly, the Bacillus species was the most sensitive, judged by the range of inhibition of growth by insecticides.

- Species Recommended for use in Aquatic Bioassay Tests

Some tolerant and sensitive species were recommended for use in aquatic bioassay tests by Arthur Scheier (Academy of Natural Sciences of Philadelphia).

Among the fish suggested were:

- o the sensitive brook trout - Salvelinus fontinalis
- o the more tolerant free-swimming bluegill - Lepomis macrochirus
- o the tolerant scavenger channel catfish - Ictalurus punctatus

TABLE 3.3.22 COMPARISON OF LETHAL CONCENTRATIONS OF POLLUTANTS ON TETRAHYMENA AND OTHER AQUATIC ORGANISMS (McKee and Wolf, 1963)

Compound	Water condition	Organism	Time	Concentration (mg l^{-1})	*Tetrahymena* data from present study Water condition	Concentration (mg l^{-1})‡
Mercuric chloride	Unknown	Minnows	42 min	10	Soft†	3.12
Mercuric chloride					Hard§	1.85
Zinc sulfate	Distilled	Minnows	3.33 h	400	Distilled	5.77
Lead nitrate	"Soft"‖	Fathead minnows	96 h	3.2‡	Soft	37.75
Lead nitrate	"Hard"‖	Fathead minnows	96 h	100‡	Hard	250
Cobalt sulfate	Unknown	Stickleback	Unknown	10	Distilled	4.08
Cadmium sulfate	Distilled	Minnows	3 h	1042	Distilled	0.84

* McKee and Wolf (1963).

† Distilled water containing 20 mg l^{-1} calcium carbonate.

‡ Tolerance limit median (concentration which kills 50 per cent of the organisms in 96 h).

§ Distilled water containing 400 mg l^{-1} calcium carbonate.

‖ Calcium carbonate concentration not specified.

TABLE 3.3.23 INHIBITION OF GROWTH OF ESTUARINE BACTERIA IN NUTRIENT SEAWATER MEDIUM BY PCB'S (Bourquin et al., 1975)

C[illegible]RL Culture No.	Gram Reaction & Morphology	Genus	Aroclor^{R} 1242(mg) 0.1	0.25	0.5	Aroclor^{R} 1016(mg) 0.1	0.25	0.5
3	+ ROD	Unknown	++	++	+++	++	++	+++
21	- ROD	Unknown	++	++	+++	++	++	+++
35	- ROD	Flavobacterium sp.	++	++	++	+	+++	+++
39	- COCCOID	Unknown	++	+++	+++	++	+++	+++
53	- ROD	Unknown	++	+++	+++	+++	+++	+++
54	+ ROD	Bacillus sp.	+++	+++	+++	+	+++	+++
7	+ ROD	Bacillus sp.	+	+	+	X	+	++
9	+ ROD	Bacillus sp.	+	++	+++	+	++	+++
31	- ROD	Unknown	+	+	+++	+	+	+++
60	+ ROD	Bacillus sp.	+	+	+	++	++	++
86	- ROD	Flavobacterium sp.	+	+	+	X	+	+
100	- ROD	Pseudomonas sp.	+	++	++	+	++	++
8	+ ROD	Corynebacterium sp.	X	+	++	X	+	++
11	- ROD	Achromobacter sp.	X	+	++	X	+	++
42	+ COCCUS	Micrococcus sp.	X	+	++	X	+	++
44	+ COCCUS	Micrococcus sp.	X	+	+	—	X	+
93	+ ROD	Unknown	X	+	+	—	X	+
43	+ COCCUS	Micrococcus sp.	—	+	++	—	+	++
5	- COCCOID	Serratia sp.	—		++	—		++
13	- ROD	Achromobacter sp.	—	—	++	—	—	++
28	- ROD	Achromobacter sp.	—	—	++	—	—	++
32	+ ROD	Corynebacterium sp.	—	∓	+	—	—	+
41	- COCCOID	Unknown	—		++	—	X	++
67	- ROD	Achromobacter sp.	—	∓	+	—	—	+
69	- ROD	Unknown	—	—	++	—	—	++

Degree of sensitivity: +++(18-20 mm zone), ++(16-18 mm), +(14-16 mm), X(slightly), —(not sensitive).

TABLE 3.3.24 THE EFFECTS OF ORGANOCHLORINE INSECTICIDES ON BACTERIAL GROWTH (Trudgill et al, 1971)

Micro-organism	Insecticide									
	Bandane	Chlordane (V)	Chlordane (L)	Heptachlor	Dieldrin	Aldrin	Endrin	Telodrin	γ-HCH	DDT
					Gram-positive					
Bacillus megaterium	-	-	-	-	-	-	-	-	-	-
B. subtilis	-	-	-	-	-	-	-	-	+	+
Streptomyces antibioticus			-	-	-	-	-	-	-	+
Nocardia sp. B	-	-	-	-	-	-	-	-	(+)	+
Corynebacterium sp. TI	-	-	-	-	-	-	-	+	+	+
B. cereus	-	-	-	-	-	-	-	(+)	+	+
Nocardia sp. A	-	-	-	-	-	-	+	+	+	+
Microbacterium flavum	-	-	-	-	-	-	+	+	+	+
Micrococcus lysodeikticus	-	-	-	-	-	-	+	+	+	+
Staphylococcus albus	-	-	-	-	-	-	+	+	+	+
Staphylococcus aureus	-	-	-	-	-	+	+	+	+	+
Sarcina lutea	-	-	(+)	+	+	+	+	+	+	+
Streptococcus faecalis	-	-								
					Gram-variable					
Arthrobacter simplex	-	-	-	-	-	+	+	+	+	+
Pseudomonas iodinum		-	-							
					Gram-negative					
Achromobacter butyri	+	+	+	+	+	+	+	+	+	+
Achromobacter sp. PC4	+	+	+	+	+	+	+	+	+	+
Escherichia coli	+	+	+	+	+	+	+	+	+	+
Klebsiella aerogenes	+	+	+	+	+	+	+	+	+	+
P. acidivorans	+	+	+	+	+	+	+	+	+	+
P. aureofaciens	+	+	+	+	+	+	(+)	+	+	+
P. dehalogens	+	+	+	+	+	+	+	+	+	+
P. fluorescens	+	+	+	+	+	+	+	+	+	+
P. fluorescens	+	+	+	+	+	+	+	+	+	+
P. multivorans	+	+	+	+	+	+	+	+	+	+
P. putida	+	+	+	+	+	(+)	+	+	+	+

+, Growth not inhibited; (+), growth slightly inhibited; -, growth severely or completely inhibited.

Among the Invertebrates were:

- the mayfly - Isonychia bicolor
- the waterflea - Daphnia pulex
- two snails:
  - the sensitive gilled snail - Amnicola limosa
  - the tolerant pulmonate snail - Physa heterostropha

Algal species suggested by Scheier are: the diatoms - Nitzschia closterium and Navicula seminulum. Mount (1968) lists some twenty fish species which have merit as bioassay test organisms (Table 3.3.25) and recently U.S. EPA (1975a) has listed recommended species for general bioassay use (Table 3.3.26).

TABLE 3.3.25 FISH SPECIES RECOMMENDED FOR USE IN AQUATIC BIOASSAY TESTS (Mount, 1968)

Common Name	Genus and Species
Threadfin shad	*Dorosoma petenense*
Brook trout	*Salvelinus fontinalis*
Rainbow trout	*Salmo gairdneri*
Northern pike	*Esox lucius*
Emerald shiner	*Notropis atherinoides*
Fathead minnow	*Pimephales promelas*
White sucker	*Catostomus commersoni*
Channel catfish	*Ictalurus punctatus*
White bass	*Morone chrysops*
Bluegill	*Lepomis macrochirus*
Largemouth bass	*Micropterus salmonides*
Yellow perch	*Perca flavescens*
Limited Distribution	
Coho salmon	*Oncorhynchus kisutch*
Lake trout	*Salvelinus namaycush*
Lake herring	*Coregonus artedii*
Mountain whitefish	*Prosopium williamsoni*
American smelt	*Osmerus mordax*
Smallmouth bass	*Micropterus dolomieui*
Walleye	*Stizostedion vitreum*

TABLE 3.3.26 RECOMMENDED SPECIES AND TEST TEMPERATURES (U.S. EPA, 1975)

Recommended species	Recommended test temperature (°C)
Freshwater	
Vertebrates	
Coho salmon, *Oncorhynchus kisutch*	12
Rainbow trout, *Salmo gairdneri*	12
Brook trout, *Salvelinus fontinalis*	12
Goldfish, *Carassius auratus*	22
Fathead minnow, *Pimephales promelas*	22
Channel catfish, *Ictalurus punctatus*	22
Bluegill, *Lepomis macrochirus*	22
Invertebrates	
Daphnids, *Daphnia magna* or *D. pulex*	17
Amphipods, *Gammarus lacustris*, *G. fasciatus*,	17
or *G. pseudolimnaeus*	17
Crayfish, *Orconectes* species, *Cambarus* species	22
Procambarus species, or *Pacifastacus leniusculus*	22
Stoneflies, *Pteronarcys* species	12
Mayflies, *Baetis* species or *Ephemerella* species	17
Mayflies, *Hexagenia limbata* or *H. bilinata*	22
Midges, *Chironomus* species	22
Marine and estuarine	
Vertebrates	
Sheepshead minnow, *Cyprinodon variegatus*	22
Mummichog, *Fundulus heteroclitus*	22
Longnose killifish, *Fundulus similis*	22
Silverside, *Menidia* species	22
Threespine stickleback, *Casterosteus aculeatus*	22
Pinfish, *Lagodon rhomboides*	22
Spot, *Leiostomus xanthurus*	12
Shiner perch, *Cymatogaster aggregata*	12
Pacific staghorn sculpin, *Leptocottus armatus*	12
Sanddab, *Citharichthys stigmaeus*	12
Flounder, *Paralichthys dentatus*, *P. lethostigma*	22
English sole, *Parophrys vetulus*	12
Invertebrates	
Shrimp, *Panaeus setiferus*, *P. duorarun* *P. aztecus*	22
Grass shrimp, *Palaemonetes* species	22
Shrimp, *Crangon* species	22
Oceanic shrimp, *Pandalus jordani*	12
Blue crab, *Callinectes sapidus*	22
Dungeness crab, *Cancer magister*	12

- Source and Size of Test Organisms

Bioassay organisms are obtained from one of two sources: natural sources such as lakes or streams, or from commercial suppliers. Organisms obtained from natural sources are generally preferred because they represent the condition of naturally occurring organisms, especially if the organisms are from the water body under study.

However, because their previous exposure to various chemicals is not readily known, performance of bioassay analyses on these organisms may, on occasion, lead to erroneous results. Another disadvantage is that because these organisms must be captured, the source of supply is not always assured. Specimens obtained from commercial suppliers have the advantage that they are usually from sources where the history of exposure is known. A disadvantage with the supply house organisms is that they often come from sources quite different from the water being assayed; even the same species from different sources may have quite different susceptibility to test materials. Additionally, some of these organisms have been inbred, resulting in various strains that are ideal for test accuracy and reproducibility (Lenon, 1967), but data obtained may be difficult to apply to natural populations of the same species.

Organisms captured by electroshocking should not be used. All organisms in a test should be from the same source and be as healthy and uniform in size and age as possible. Whenever trout are to be used, certified disease-free fish (free of infectious pancreatic necrosis, furunculosis, kidney disease, and whirling disease) should be obtained. Freshwater amphipods, daphnids, and midge larvae should be reared in the testing facility from laboratory cultures. Daphnids from cultures in which ephippia are being produced should not be used (U.S. EPA, 1975a).

The size of the test organism is a major consideration. The organism should not be so small that it is difficult to observe and contain in the test cell, especially if the tests incorporate large continuous flow apparatus with a continuous discharge of test water. This notion is changing with increased emphasis on diatoms, protozoans, and small invertebrates as bioassay organisms. Test species, on the other hand, should not be so large as to limit their activity, body functions, and handling advantages in the test units (Rand et al., 1975; Sprague, 1971).

o Fish

Very young (not yet actively feeding), spawning or recently spent fish should not be used. The use of fish that weigh between 0.5 and 5.0 g each is usually desirable. Embryos and newly-hatched fish are sometimes more sensitive than older ones and can be tested if appropriate precautions are taken. The standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than twice that of the shortest fish (U.S. EPA, 1975a).

o Invertebrates

Immature organisms should be used whenever possible. Daphnids should be in the first instar; amphipods, stoneflies and mayflies in an early instar; and midges in the second and third instar (U.S. EPA, 1975a).

o Amphibians

Young larvae should be used whenever possible (U.S. EPA, 1975a).

o Shrimps

Larval stages should be used.

o Mollusks

For mussel and oyster, adults or juveniles should be used.

o Lobster

Adults or juveniles should be used (Rand et al., 1975).

- Care and Handling

It is of utmost importance for bioassay studies that the test animals be kept in excellent condition before the test. Never allow abrupt changes in environmental conditions. In general, aquatic organisms should not be subjected to more than a 3°C change in water temperature in any 12-hour period. During transport to the laboratory, do not crowd the organisms, supply plenty of oxygen and maintain a favorable temperature (U.S. EPA, 1975a).

The dissolved oxygen concentration must be maintained between 60% to 100% of the saturation concentration; gentle aeration may be used if necessary (U.S. EPA, 1975a). Provide adequate flow-through water so that the dissolved oxygen, pH, carbon dioxide, salinity, hardness, and other characteristics are favorable. Generally use a flow-through rate equivalent from 6 to 16 tank volumes per day (Rand et al., 1975).

Test organisms should be fed at least once a day and the tank scrubbed at least twice a week. Remove within 24 hours all uneaten food that collects on the bottom or in corners. Recommended diets and feeding schedules are given in Table 3.3.27 (Lenon, 1967).

Shield the tank with curtains or some other means to protect the organisms from nearby movements and noise. Provide photoperiods and light intensities favorable to the organisms. In long-term studies for those species that require annual light cycle photoperiods, simulate the natural seasonal daylight and darkness period with appropriate twilight periods. Make adjustments in photoperiod on the first and fifteenth of every test month.

TABLE 3.3.27 DIETS AND FEEDING SCHEDULE (IN DAYS PER WEEK*) AT THE FISH CONTROL LABORATORY FOR VARIOUS BIOASSAY SPECIES (Lenon, 1967)

Species	Commercial trout pellets	Oregon moist pellets	Liver	Live *Daphnia*	Frozen brine shrimp	Other
Rainbow trout (*Salmo gairdneri*)	7	1	2			
Brown trout (*Salmo trutta*)	7	2	5			
Brook trout (*Salvelinus fontinalis*)	7		5			
Lake trout (*Salvelinus namaycush*)	7	2	5			
Northern pike (*Esox lucius*)			2	7	2	2(a)
Goldfish (*Carassius auratus*)	7		2			
Carp (*Cyprinus carpio*)	7		2			
Fathead minnow (*Pimephales promelas*)	7		5			
White sucker (*Catostomus commersoni*)	7	1	5			2(b,c)
Black bullhead (*Ictalurus melas*)	7	1	5			2(b,c)
Channel catfish (*Ictalurus punctatus*)	5	1	5			2(b)
Green sunfish (*Lepomis cyanellus*)	7		5			
Bluegill (*Lepomis macrochirus*)			5	2	7	
Smallmouth bass (*Micropterus dolomieui*)			5	7	2	2(a)
Largemouth bass (*Micropterus salmoides*)		2	5	2	7	2(a)
Yellow perch (*Perca flavescens*)		2	5	2	2	
Walleye (*Stizostedion vitreum*)		2	5	7	2	2(a)

*Large size fish are not fed on weekends.

(a) minnows (*Pimephales promelas*).

(b) soybean meal.

(c) torula yeast.

For details see Table 3.3.28 Test Photoperiod for Brook Trout, Partial Life Cycle (Rand et al., 1975). In short-term tests, standard photoperiod of 14 hour light, 10 hour dark is suggested, but often the usual laboratory lighting is adequate.

Hold field collected animals in quarantine for at least seven days to observe them for disease, stress, physical damage or mortality. If more than 10% of the collected animals die after the second day or they are heavily parasitized or diseased and the problem cannot be controlled, destroy the lot and clean and sterilize all containers and equipment used. At the end of the quarantine period, transfer the test organisms that appear to be disease-free to the regular laboratory stock tanks. The handling should be done as gently, carefully, and quickly as possible. Organisms that touch a dry surface or are dropped or injured during handling must be discarded. Small dipnets are best for handling small fish. Smooth glass tubes with rubber bulbs should be used for transferring smaller organisms such as

TABLE 3.3.28 TEST (EVANSVILLE, INDIANA) PHOTOPERIOD FOR BROOK TROUT, PARTIAL LIFE CYCLE (Rand et al, 1975)

Dawn to Dusk Time	Date	Day Length (hr & min)	
6:00-6:15	Mar. 1	12:15	Juvenile-adult exposure
6:00-7:00	15	13:00	
6:00-7:30	Apr. 1	13:30	
6:00-8:15	15	14:15	
6:00-8:45	May 1	14:45	
6:00-9:15	15	15:15	
6:00-9:30	June 1	15:30	
6:00-9:45	15	15:45	
6:00-9:45	July 1	15:45	
6:00-9:30	15	15:30	
6:00-9:00	Aug. 1	15:00	
6:00-8:30	15	14:30	
6:00-8:00	Sept. 1	14:00	
6:00-7:30	15	13:30	
6:00-6:45	Oct. 1	12:45	Spawning and egg incubation
6:00-6:15	15	12:15	
6:00-5:30	Nov. 1	11:30	
6:00-5:00	15	11:00	
6:00-4:45	Dec. 1	10:45	Alevin-juvenile exposure
6:00-4:30	15	10:30	
6:00-4:30	Jan. 1	10:30	
6:00-4:45	15	10:45	
6:00-5:15	Feb. 1	11:15	
6:00-5:45	15	11:45	

Daphnids and midge larvae. Equipment used to handle aquatic organisms should be sterilized between uses with an Iodophor, 200 mg of Hypochlorite/liter or 30% Formalin plus 1% Benzalkonium chloride (U.S. EPA, 1975a).

Generally organisms should not be treated for disease during the first 16 hours after they arrive at the facility because they are probably stressed due to collection or transportation and some may have been treated during transit. However, immediate treatment is necessary in some situations, such as treatment of bluegills for columnaris during hot weather (U.S. EPA 1975a). To reduce mortality and to avoid introduction of disease into stock tanks, treat with a wide-spectrum antibiotic immediately after collection or during transport. Holding in tetracycline (15mg/l) for 24 to 48 hours can be very helpful (Rand et al., 1975).

Table 3.3.29 gives recommended prophylactic and therapeutic treatments for freshwater fish (U.S. EPA, 1975a).

TABLE 3.3.29 RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH[a] (U.S. EPA, 1975a)

Disease	Chemical	Concentration (mg/l)	Application
External bacteria	Benzalkonium chloride (Hyamine 1622^R)	1-2 AI[b]	30-60 min[c]
	Nitrofurazone (water mix)	3-5 AI	30-60 min[c]
	Neomycin sulfate	25	30-60 min[c]
	Oxytetracycline hydrochloride (water soluble)	25 AI	30-60 min[c]
Monogenetic trematodes, fungi, and external protozoans[d]	Formalin plus zinc-free malachite green oxalate	25 0.1	1-2 hours[c]
	Formalin	150-250	30-60 min[c]
	Potassium permanganate	2-6	30-60 min[c]
	Sodium chloride	15000-30000 2000-4000	5-10 min dip[e,c]
	Dexon^R (35% AI)	20	30-60 min[c]
Parasitic copepods	Trichlorfon (Masoten^R)	0.25 AI	f

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[a] These recommendations do not imply that these treatments have been cleared or registered for these uses. These treatments should be used only on fish intended for research, and researchers are cautioned to test treatments on small lots of fish before making large-scale applications. Before a treatment is used, additional information should be obtained from sources such as: Davis (1954), Hoffman and Meyer (1974), Reichenbach-Klinke and Elkan (1972), Snieszko (1970) and Van Duijn (1973).

[b] Active ingredient.

[c] Treatment may be accomplished by:

- o Transferring the fish to a static treatment tank and back to holding tank
- o Temporarily stopping the flow in a flow-through system, treating the fish in a static manner and then resuming the flow to flush out the chemical
- o Continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or the technique of Mount and Brungs (1967).

d One treatment is usually sufficient except for "Ich", which must be treated daily or every other day until no sign of the protozoans remains. This may take 4 to 5 weeks at 5 to 10°C and 11 to 13 days at 15 to 21°C. A temperature of 32°C is lethal to "Ich" in one week.

e Minimum of 24 hours but may be continued indefinitely.

f Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for over 4 weeks and should not be used above 27°C.

Freshwater invertebrates and amphibians must have been in holding tanks for at least 10 days and fish for at least 14 days before they are used for basic tests; all test organisms must have been in holding tanks for at least four days before they are used for effluent tests. They should be held under stable condition of temperature and water quality in uncontaminated, constant-quality water in a flow-through system with a flow rate at least two water volumes per day. Water from a well or spring should be used for freshwater organisms whenever possible. Only as a last resort should a dechlorinated water be used. The cold-water freshwater organisms are best held between 5°C to 15°C, usually well below 15°C. Hold warm-water organisms at temperature between 10°C to 25°C depending on the season. Hold aquatic invertebrates within the temperature range of the water from which they were obtained unless they are being acclimated for special temperatures or other tests. If possible, follow the natural variations in temperature. During long holding periods, hold most test organisms in the lower range of favorable temperature rather than at higher temperature because the metabolic rate and the number and severity of disease outbreaks are reduced in the cooler water.

The acclimation of the test organisms to the test condition begins from one to two weeks before they are to be used in bioassays. There should be few or no deaths due to parasites or diseases during this period. Use only those groups of organisms that are free from parasitic infection and diseases and in which the mortality is less than 10% during the laboratory holding period. Never allow abrupt changes in environmental conditions; often it is helpful to follow the natural seasonal variations in environmental conditions such as temperature and the seasonal daylight patterns. There should be no supersaturation of gases. If the organisms in the holding tank are not exposed to the same conditions as those to be used in the bioassays, gradually acclimate them to temperature and other conditions to which they will be exposed in the actual bioassays. Freshwater amphipods, daphnids, and midge larvae should be acclimated to water quality and temperature by rearing them in dilution water at the test temperature. Other organisms can be acclimated (in a flow-through system with a flow rate of at least two water volumes per day for flow-through tests) simultaneously to the dilution water and test temperature by transferring the appropriate number of similar-length individuals from a holding tank to an acclimation tank. They should be acclimated to the dilution water by gradually changing the water in the acclimation tank from 100% holding water to 100% dilution water over a period of 2 or more days for basic tests and for at least 24 hours for effluent tests before they are used for test. For basic test, water that may be contaminated by undesirable microorganisms should be passed through an ultraviolet sterilizer and the un-ionized ammonia concentration in the acclimation tanks should be less than 20 µg/l. They should be acclimated to the test temperature by changing the water temperature at a rate not to exceed 3°C within 72 hours for basic test and not to exceed 3°C within 24 hours for effluent tests until the allowable test temperature range is reached. They must be maintained for at least 48 hours for basic tests and 24 hours for effluent tests at the allowable test temperature range before tests are begun with them. Longer acclimation times are generally desirable.

A group of organisms must not be used for a test if the individuals appear to be diseased or otherwise stressed or if more than 3% for basic tests or 5% for effluent tests die during the 48 hours immediately prior to the beginning of the test. If a group fails to meet these criteria, all individuals must be either discarded or treated, held an additional 10 days for basic tests or 4 days for effluent tests, and reacclimated if necessary.

Young amphibian larvae and fish that have been actively feeding for less than about 20 days, amphipods, daphnids, and midge larvae must be fed, and all other insects may be fed, up to the beginning of the test. For basic tests all other amphibian larvae and fish over 0.5 g each must not be fed for 96 hours and all other invertebrates over 0.5 g each must not be fed for 48 hours before the beginning of the test. For effluent test, all other amphibian larvae, fish, and invertebrate over 0.5 g each must not be fed for 48 hours before the beginning of the test (U.S. EPA, 1975a).

3.3.1.5 Design of Experiment--

The precision of a test procedure depends on the following factors (Rand et al., 1975):

- The variability of the organisms in their response
- The number of organisms exposed to each test concentration
- The number of replicates being made
- The test substance to which the organisms are exposed
- How close the mid-concentration tested happened to be to the LC50 and how closely the concentrations of the test substance solutions cluster round the LC50 concentration

For a given test under similar conditions, increasing the number of test organisms increases the precision. The use of more organisms and replicate test containers for each test substance concentration is often desirable to reduce variability (U.S. EPA, 1975a).

The number of organisms to be exposed in each test concentration is governed by a number of considerations:

- the size of the organisms
- the expected apparent normal mortality
- the extent of cannibalism
- the availability of dilution water, toxicant, and test organisms
- the desired precision of the estimate of the toxicity of the test material.

Replicates must be true replicates with no water connection between the test containers. If replicates are used, random assignment of one test container for each test concentration in a row followed by random assignment of a second test container in a second row or an extension of the same row is recommended rather than total randomization (U.S. EPA, 1975a).

A representative sample of the test organisms should be impartially distributed to the test chambers, either by adding one (if there are to be less than 11 organisms per container) or two (if there are to be more than 11 organisms per container) test organisms to each chamber, and then adding one or two more, and repeating the process until each test chamber has the desired number of test organisms in it. Alternatively, the organisms can be assigned either by random assignment of one organism to each test chamber, random assignment of a second organism to each test chamber, etc., or by total randomization. It is often convenient to assign organisms to other containers and then add them to the test chambers all at once.

Every test requires a control in which the same dilution water, conditions, procedures, and organisms are used as in the remainder of the test. If any additive is present in any of the test chambers, an additive control is also required. This additive control is treated the same as the regular control except that the highest amount of additive present in any other test chamber is added to this test chamber. A test is not acceptable if more than 10% of the organisms die in any control in a test determining an LC50 or show the effect in a test determining an EC50. It is desirable to repeat the test at a later time to obtain information on the reproducibility of the results of the test (U.S. EPA, 1975a).

3.3.1.6 Test Methods--

Toxicity tests with aquatic organisms should be conducted according to uniform, detailed methods whenever possible to maximize the number of reliable comparisons that can be made concerning relative toxicity and relative sensitivity. Tests shall include control groups to determine if any observed effects have developed or occurred independent of the test substances. The control group shall be maintained in the same manner as the test group (Anon., 1977). One or more control treatments should be used to provide a measure of the acceptability of the test by giving some indication of the healthiness of the test organisms and the suitability of the dilution water, test conditions, and handling procedures. Widespread adoption of uniform methods will promote the accumulation of comparable data and increase its effective use (U.S. EPA, 1975a).

Whenever toxicity tests are conducted with aquatic organisms, the methods recommended by U.S. EPA (1975a) should be followed as closely as possible. Use of these methods for special purposes may require modifications or specification of additional details, such as choosing one particular species. Since not all details are covered in these methods, the successful execution of these methods will require some training or experience in aquatic toxicology or aquatic biology or both. It is essential to conduct tests so that they meet specific needs but these methods should cover most situations (U.S. EPA, 1975a).

Some novel bioassay procedures that have been suggested are outlined below:

Roberts (1975)	Byssus formation in mussels was sensitive to pesticides and PCP's. Byssogenesis test was proposed as a rapid and convenient technique for routine screening of potential marine pollutants.
Walker et al., (1975)	Barnacles were suggested as possible indicators of Zn pollution based on the studies of Zn accumulation in *Balanus*, *Eliminus*, and *Lepas*.
Boree (1975)	A photomicrographic method was proposed to determine the degree of response of the protozoan *Tetrahymena pyriformis* to metal levels which was similar to that of the bluegill sunfish.
Jensen (1976)	A procedure based on the hatching rate of eggs of the brine shrimp *Artemia Salina* revealed the convenience of a bioassay organism that could be stored dry in the laboratory. The method offers an easy way to get information of the toxicity of a particular matter. The experimental results of the hatching tests show a characteristic graph typical of many toxicity tests.
Baudouin and Scoppa (1975)	Nucleic acids were used as indicators of biomass in mixed planktonic populations. DNA and RNA showed large variations among different planktonic species, between zooplankton and phytoplankton and among seasons.
Canton et al, (1975)	A tentative method for deriving an EC50 (ecological limit) was proposed. The criteria included mortality, immobilization, growth, reproduction, histopathologic changes, and enzyme activities. The procedure was based on short-and long-term toxicity studies with d-hexachlorocyclohexane and a variety of organisms including algae, crustacea, and fish.

The following are specific laboratory procedures that are used to insure maintenance of sample integrity and treatment.

- Receptacles, pipettes, and other instruments used for handling specimens must be kept separate from those used for chemicals.

- Specimens generated from field investigations are designated by the field data sheet number; specimens generated from laboratory investigations are given a data sheet number) these numbers are then entered in a log book

- Fixation (within 24 hours)

Davidson's fixative has been recommended as follows:

Formalin	20 parts
Glycerin	10 parts
Ethyl alcohol 95%	30 parts
Glacial acetic acid	10 parts
Distilled water	30 parts

Since the nature of the fixing agent has considerable effect upon the affinity of the structures for various stains, special staining procedures require the use of different fixatives. When fixatives other than Davidson's are used (i.e., Formalin) the specimens are washed in running water or alcohol to remove the fixative before proceeding with dehydration.

- Preservation

To prevent disintegration or alteration of important constituents of fixed tissue, specimens are kept in a solution of one part glycerol to nine parts of 70% alcohol. Since the staining qualities of tissues begin to deteriorate after the tissues have remained in alcohol for weeks or months, specimens which are eventually to be stained and mounted are transferred to glycerol for storage.

- Decalcification

Specimens which contain deposits of calcium salts which are too hard to be cut with a microtome knife are decalcified with a 3% solution of hydrochloric acid in 70% alcohol. This causes no serious damage to tissues. The tissues are then placed in neutral 70% alcohol.

- Dehydration

To prevent violent diffusion currents which would cause the collapse of cavities or the distortion of specimens, a graded series of alcohol concentrations is used.

- Embedding

The dehydration and embedding steps are carried out using an Autotechnicon. The procedure is as follows:

70% alcohol	1 hour
95% alcohol	1 hour
100% alcohol	1 hour
100% alcohol	1 hour
1:1 benzene: 100% alcohol	1 hour
100% benzene	1 hour
100% benzene	1 hour
paraffin	2 hours
paraffin	2 hours

Vacuum infiltration - 20 minutes at 15 psi, or 15 to 20 minutes at 12 psi for tissues that are relatively delicate. Each specimen is placed in its own labelled tissue capsule before being placed in the Autotechnicon. Once a day the beakers containing the 100% alcohol and the 95% alcohol are changed. Every three to five days the beaker containing the 1:1 benzene alcohol is changed. If the instrument sits for a few days, all the solutions are changed except the paraffins.

The paraffin in the vacuum infiltrator is changed once a week if used frequently. After complete paraffin infiltration, the specimens are placed in a labelled embedding mold and made into paraffin blocks. If the paraffin blocks are not sectioned immediately, they are labelled and stored in a specimen cabinet.

Once slides are finished they are stored in labelled slide boxes.

Staining (trichome type staining)

1. 100% xylene - 3 min (50 to 56°C) (3 changes)
2. 100% ethanol - 3 min (room temp.) (2 changes)
3. 95% ethanol - 3 min (room temp.)
4. 50% ethanol - 3 min (room temp.)
5. 10% ethanol - 3 min (room temp.)
6. distilled water - 3 min (room temp.) (2 changes)
7. 4% ferric ammonium sulfate - 15 min (50 to 56°C)
8. tap water - quick rinse to remove any excess
9. hematoxylin stain - 15 min (50 to 56°C)

*10. distilled water - couple of rinses
11. destaining - 4% ferric ammonium sulfate, room temp. - about 1.5 min
12. tap water - 3 to 4 min
13. basic ethanol - 30 sec to 1 min
*14. water bath
15. acid fuchsin - 4 quick dips
16. distilled water - 4 quick dips
17. drain on paper towel to remove excess liquid
18. 1% phosphomolybdic acid - 5 min (room temp.)

19. drain for 30 sec
20. aniline blue stain - 1.5 min
21. drain - 1 to 2 min
22. destaining - 1% acetic acid - 1 min (4 changes)
23. dehydration - 1% acetic acid in acetone - 1 min (3 changes)
24. 100% xylene - 1 to 2 min
25. 100% xylene - (keep out of sunlight) - can keep here 24 to 48 hours
26. mount - use #1 coverslip (try to flatten out the cover slip as much as possible)

* The slides can be held at these steps for at least 24 hours.

3.3.1.8 Data Handling--

Data Collection

For maintaining a quality bioassay capability, all information about conduct of the experiment collected should be recorded on either a Bioassay Biota Log Sheet (Figure 3.3.3) or a Bioassay Water Quality Log Sheet (Figure 3.3.4) (U.S.EPA, 1975b). A typical schedule of checks and maintenance during studies carried out in tanks could look as follows (U.S. EPA 1975b).

- Daily: check all tanks for signs of disease, abnormal organisms behavior and dead organisms.
- Mon., Wed., Fri., or every other day: feed organisms and remove unconsumed food within one hour.
- Filter cleaning: high volume pump - once every three weeks Dyno flow - once a week. Filter may need change sooner if tank appears cloudy or going bad.
- Water exchange: Monthly (drain half of the water, then add distilled water and chemicals).

Similarly, a typical schedule of checks to be performed with holding tanks and experimental units could look as follows (U.S. EPA, 1975b):

- Holding tanks: Determine daily air temperature, water temperature, and dissolved oxygen. Determine monthly pH, alkalinity, hardness, calcium, conductivity, and salinity.
- Experimental units: Determine every 24 hr dissolved oxygen, pH, air temperature, water temperature, and conductivity. Determine at end of test hardness, calcium, alkalinity and salinity.

Note: all water samples should be taken at mid-depth.

WATER TYPE: SALT F-WATER
(CIRCLE ONE)
TANK TYPE: HOLDING EXPERIMENTAL
(CIRCLE ONE)

			BIOTA				NOTES	MAINTENANCE					
										WATER CHANGE		
DATE	TIME	TANK SECTION	SPECIES	CHANGE IN NO	BAL	REASON FOR CHANGE	BEHAVIOR, APPEARANCE CONDITION	FEEDING TYPE	FILTER CHANGE	TYPE	AMOUNT	REASON

Figure 3.3.3 Bioassay biota log (U.S. EPA, 1975b)

DATE	TIME	TANK SECTION	°C TEMP		pH	mg/l AS $CaCO_3$				O_2 mg/l	COND $\frac{\mu mho}{cm}$	SAL %o	REMARKS
			AIR	W		TOTAL ACIDITY	ALK	HARD	Ca				

Figure 3.3.4 Bioassay water quality log (U.S. EPA, 1975b)

SERIES: ______________ COMPANY: ______________ DATE: __________

TECHNICIAN: ______________________________ STARTING HOUR: __________

MATERIAL BEING TESTED: ______________________________

SOURCE: ______________________________

SOURCE OF DILUTION WATER: ______________________________

TEST SPECIES: ______________________________ TEMP. RANGE: __________

NO. INDIVIDUALS PER CONCENTRATION: ______________________________

START

DILUTION:						CONTROL
DO						
pH						
HARDNESS						
OTHER						

24 HOURS

NO SURVIVING						
% SURVIVAL						
DO						
pH						
OTHER						

48 HOURS

NO SURVIVING						
% SURVIVAL						
DO						
pH						
OTHER						

72 HOURS

NO SURVIVING						
% SURVIVAL						
DO						
pH						
OTHER						

96 HOURS

NO SURVIVING						
% SURVIVAL						
DO						
pH						
OTHER						

Figure 3.3.5 Bioassay record sheet (U.S. EPA, 1975b)

Results of the experiment should be recorded on a form similar to Figure 3.3.5. In addition to toxicity data, the following information should be recorded:

- name of method, investigator, and laboratory, and date test was conducted
- detailed description of the toxicant or effluent
- source of dilution water
- detailed information about the test organism
- a description of the experimental design and test chambers - the depth and volume of solution in the test chambers, flow rate, etc.
- definition of the criterion used to determine the effects and a summary of general observations on other effects or symptoms
- percentage of organisms that died or showed the effect in the control treatment
- the average and range of the acclimation temperature; test temperature
- methods used for and the results of, all chemical analyses of water quality and toxicant concentrations
- anything unusual about the test; any deviation from these methods and any other relevant information (U.S. EPA, 1975a).

Photography may be used to document organisms response, test set-up and physical appearance of waste concentrations (U.S. EPA, 1975b).

- Biological Response

The most common toxicity test response with aquatic animals is the mortality which is counted to obtain information about a median lethal concentration (LC50). The data produced by the test generally consist of the percentages or organisms that are killed by different concentrations of a toxicant after specified lengths of exposure. A statistical estimation method is then used to obtain the best estimate of the LC50 from the concentration mortality data for each length of exposure (Stephan, 1976).

The precision of a toxicity test is limited to a number of factors including the normal biological variation among individuals of a species. Toxicity studies with a randomly selected species cannot be expected to give accurate information on the toxicity of that material to other species and life stages or to an entire biota. A toxicity test with one species yields an accurate estimate of the toxicity only to others of that species of similar size, age and physiological condition and in water with the same characteristics and under similar test condtions (Rand et al. 1975).

In order to obtain information about the precision of the acute mortality test, replicate test must be conducted at different times in one laboratory and/or in different laboratories (Stephan, 1976).

- Statistical Estimation Method

The statistical estimation method should meet the following criteria:

- The method should be a strictly computational method
- The method should be just as useful whether or not the toxicant concentrations are in a geometric series and whether or not the complete range from 0% to 100% kill is covered
- The method should not require exposing the same number of organisms to each toxicant concentration
- The use of adjusted or assumed data should not be required for any set of data

Based on statistical considerations, the log - probit method has been highly recommended by Sprague (1969). It has the advantage of:

- Complete toxicity curves for easy interpolation of results
- An incipient LC50 instead of one for an arbitrary time
- A mathematical instead of a subject estimate of incipient LC50

It allows the toxicity of different pollutants to be compared easily and meaningfully. Analysis of results by the rapid graphic methods of Litchfield-Wilcoxon (1949) is recommended (these improvements are also suggested by Rand et al. 1965). To carry out the Litchfield-Wilcoxon procedure, actual percentage mortality in each test tank at the selected time beyond the lethal threshold is plotted on log-probit paper (Figure 3.3.6). A line is fitted to the points by eye. Its goodness of fit is estimated by a rapid chi-square value. The incipient LC50 is then read from the graph. If desired, the more formal but more time-consuming mathematical procedures of Finney (1964) may be used to estimate the incipient LC50 (Sprague, 1969).

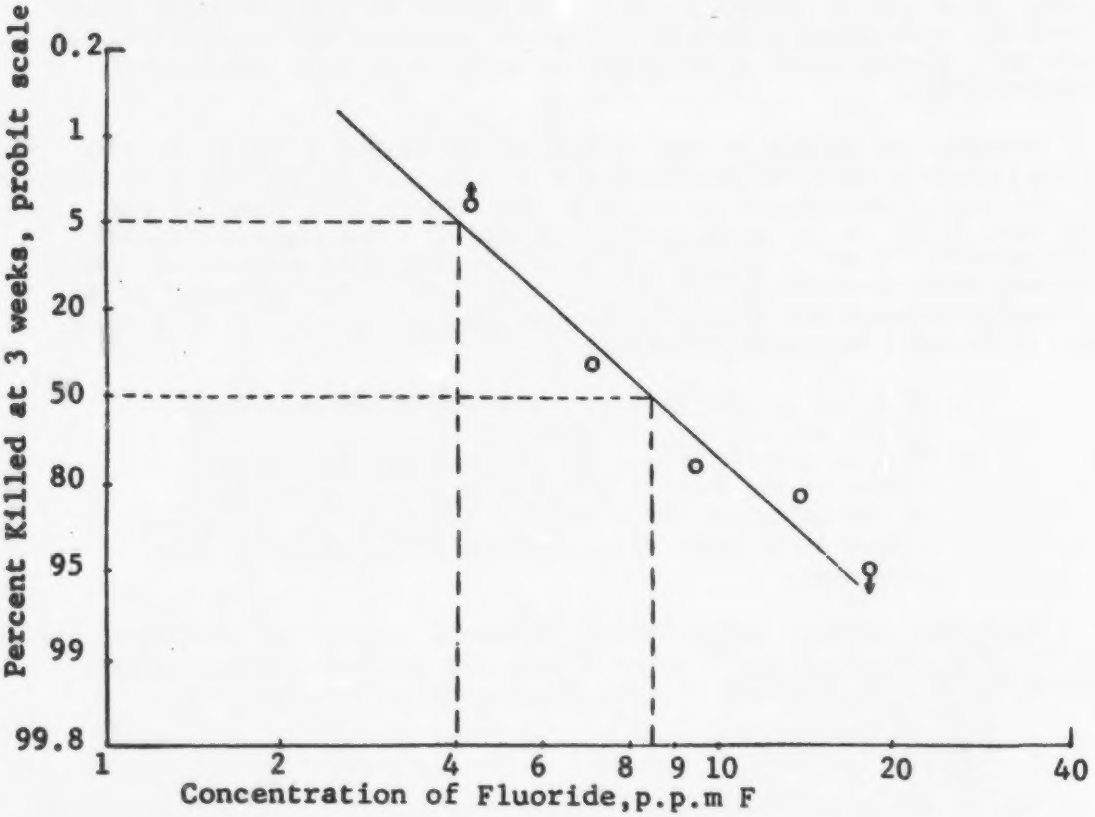

Figure 3.3.6. Estimating the median lethal concentration. In this case the incipient LC50 is estimated since the exposure time was long. Percentage response of trout is plotted on the vertical probit scale. The median lethal concentration is 8.5 mg/l and its confidence limits could be estimated as described in the text. The 5% response is also shown. From Herbert and Shurben (1964).

The moving average method can be used to calculate the best estimate of the LC50 and its 95% confidence limits for all acute mortality tests, except that confidence limits cannot be obtained if there are no partial kills.

A modification of the moving average method is the moving average angle method suggested by Bennett (1952). The purpose of using an angle transformation with binomial data is to improve linearity and to stabilize the variance, thus allowing equal weight to be given to each transformed observation.

Whenever any method is used to analyze concentration-mortality data, the logarithmic transformation should probably be used on the concentration data. If the log transformation is not used, the formula LC50 = (A+B)/2 will give the same result as the moving average method (A = the highest toxicant concentration in which none of the test organisms died and B is the lowest concentration in which all of the organisms died). The following is the recommended scheme for analyzing concentration mortality data from acute mortality tests with aquatic animals:

- o With one or more partial kills, use a moving average method and log concentration,
- o With no partial kills, use either a moving average method or the formula 1/2(A+B) to obtain an estimate of the LC50, and use A and B in place of 95% confidence limits if at least five organisms were exposed to each treatment.

Regardless of what method is used to obtain an LC50 and confidence limits, the results should always be compared with the original concentration-mortality data to determine if they are reasonable (Stephan, 1976).

- Control Mortality

Control mortality should be virtually absent. It should not be greater than 10% and preferably not more than 5%, representing an occasional weak organism in a group. Make corrections for higher mortality in controls by Abbott's formula (Rand et al., 1975). According to Stephan (1976) the use of Abbott's formula for some sublethal acute toxicity tests may be appropriate if a percentage of the test organisms consistently shows the effect in the absence of the toxicant.

3.3.2 Experimental Procedures in Aquatic Bioassay

Aquatic bioassay procedures may be categorized as:

- acute or chronic bioassay, depending on whether effects are observed in the short or the long term;

- static or flow-through bioassay, depending on whether the water in the tank is still or continuously flowing;

- basic or effluent, depending on whether the toxicant is added to the water or whether discharge water already containing the toxicant is used.

These categories are not mutually exclusive. The static procedure is most often used for acute bioassay because of its advantages in short term applications. Similarly, the flow-through procedure is most often used for chronic studies because of the advantages it has for long-term tests. Also, by the nature of the water supply, testing of effluents lends itself best to the flow-through procedure.

Whether an acute or chronic bioassay is used depends on the objectives of the experimenter and on the stage of experimentation. The acute test may by itself satisfy the aim of the experimenter or it may be used as a precursor of a chronic test. There may be a series of intermediate stages such as repeated dose and sub-chronic tests, each adding more information and building up to the long-term, usually very expensive, chronic bioassay. Whether a static or flow-through procedure is used is a matter of choice on the part of the experimenter who will use the kind of experimental set-up most suitable to his purpose. Also, whether basic treatment of the water or effluent water is used depends upon the nature of the situation being examined.

In naming an aquatic bioassay protocol, the essential descriptors are "acute" or "chronic". These terms may or may not be accompanied in the name of the test by "static" or "flow-through" because the instructions for performance of the test make the conditions of the test explicit. The terms "basic" or "effluent" do not often appear in the name but the condition which applies is apparent from the context of the test.

- Static Bioassay

In addition to its short-term characteristics, the static bioassay procedure offers the following advantages:

- it allows for testing of different toxicants in parallel
- it allows for testing of several species at the same time
- homogeneous water is used
- fewer numbers of animals are required
- lower cost
- more easily reproduced (replicated)
- requires minimum space, equipment and maintenance

The disadvantages of the procedure are:

- production of irregular concentrations if test material is volatile
- usually gives a lower LC50 reading than flow-through bioassay (Martin, 1973)
- dissolved oxygen, metabolic products and food wastes may create problems.

- Flow-through bioassay

The flow-through bioassay procedure has the following advantages:

- o It is useful when the test material is volatile, easily precipitated, or when the expression of its effects is long coming
- o can be used for life-time tests
- o more readily represents natural systems
- o good for determining response of lethality

A disadvantage is that it is more complicated and requires close attention over long periods of time.

3.3.2.1 General Factors in Aquatic Bioassay--

- Experimental design

  Usually the design consists of:

  - o One control and 5 or 6 concentrations of toxicant
  - o At least 10, but preferably 20 organisms exposed in each treatment and the control groups. The use of more organisms and replicate test chambers for each treatment is desirable, but "loading" must be avoided
  - o True replicates with no water connection
  - o Tanks and the test organisms assigned either by stratified randomization or total randomization
  - o Randomization of the treatment
  - o A control consisting of the same dilution water, conditions, procedures, and organisms as are used in the remainder of the test (U.S. EPA, 1975a).

- Dissolved oxygen concentration

Test solutions must not be aerated in the test chambers or in the toxicant delivery system. For static tests, the dissolved oxygen concentration in each test chamber must be between 60% to 100% saturation during the first 48 hours of the test and must be between 40% and 100% saturation after 48 hours. For flow-through tests, the dissolved oxygen must be between 60% and 100% saturation at all times (U.S. EPA, 1975a).

- Test Temperature

For basic tests, the test temperature must be selected from the series 7°, 12°, 17°, 22°, and 27°C. The actual test temperature must not deviate from the selected test temperature by more than 1°C at any time during the test. For aquatic invertebrates, the selected test temperature should be within 5°C of the temperature of the water from which they were obtained.

For an effluent test, the selected test temperature should be the temperature of the receiving water measured just outside the zone of influence of the effluent at noon on the day before acclimation begins, because the temperature at noon usually approximates the average temperature for the day. The actual test temperature must not deviate from the selected test temperature by more than 2°C at any time during the test (U.S. EPA, 1975a).

The suggested test temperature for vertebrates and invertebrates is as follows (U.S. EPA, 1976):

Region*	Temperature
I	20°C
II** and III	25°C
IV, VI and IX	30°C
X	15°C

- Salinity

The salinity of the test water should be that of the discharge site if effluent water is used or if artificial sea water is prepared. The salinity of any other natural sea water should be greater than or equal to 15% (U.S. EPA, 1976).

- Loading

The grams of organisms per liter of solution in the test chambers must not be so high that it affects the results of the test. The loading must be limited to insure that the concentration of dissolved oxygen and toxicant is not decreased below acceptable levels, that the organisms are not stressed due to crowding, and that the concentration of metabolic products does not increase above acceptable levels. For static tests, lower loadings must be used if necessary to maintain the concentration of dissolved oxygen above 60% saturation for the first 48 hours of the test and above 40% saturation after 48 hours. For flow-through tests, lower loadings should be used to maintain the concentration of dissolved oxygen in the dilution water above 60% at the beginning of the test, to keep unionized ammonia below 20 μg/l, and to limit to 20% the lowering of toxicant concentration because of uptake by the test organisms. In order to determine the effects of the test organisms on the dissolved oxygen concentration during effluent tests, the dissolved oxygen concentration should be measured in duplicate test chambers that do not contain test organisms (U.S. EPA, 1975a).

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* Temperature should be revised to the highest average monthly temperature of oceanic surface waters in each region.

** Puerto Rico and Virgin Islands are in Region II but should use temperatures suggested for Region IV.

- Feeding

The test organisms must not be fed while in the test chambers (U.S. EPA, 1975a).

- Range-finding

Generally, groups of five organisms are exposed to three to five widely spaced toxicant concentrations and a control for 24 to 96 hours using either the static or flow-through techniques.

Range-finding tests may often be difficult to conduct for effluents because the characteristics of the effluent and the receiving water may vary significantly within short periods of time. If a range-finding test is to be conducted with the same grab sample of the effluent with which a definitive effluent test is to be conducted, the range-finding test can last 8 hours at the most (U.S. EPA, 1975a).

- Definitive test

A definitive test must meet both of the following criteria so that the LC50 or EC50 can be calculated with reasonable accuracy:

  - o Except for the controls, the concentration of toxicant in each treatment must be at least 60% of the next higher one for basic tests and at least 50% of the next higher one for effluent tests.

  - o One treatment other than the control must have killed or affected more than 65% of the organisms. If an LC or EC near the extremes of toxicity is to be calculated, such as LC10 or EC90, at least one treatment must have killed or affected a percentage of test organisms, other than 0% and 100%, near the percentage for which the LC or EC is to be calculated. This requirement might be met in a test to determine the LC50 or an EC50, but special tests with appropriate toxicant concentrations will often be necessary (U.S. EPA, 1975a).

- Control Test

A concurrent control test should be performed along with each test of any concentration of the substance assayed or with each series of tests of different concentrations tested simultaneously (Doudoroff et al., 1951).

It should be performed in exactly the same manner as the other test, but using the diluent water alone as the medium in which the test organisms (control) are held. There should be no more than 10% mortality among the controls during the course of a test and at least 90% must remain apparently in good health. Otherwise, the results cannot be deemed reliable.

3.3.2.2 Static Bioassay--

The static test utilizes a procedure where the test or dilution water is initially dosed with the desired concentration of material and the solution is adjusted from time to time to maintain the selected concentration. While static tests are relatively easy to operate and maintain, they do not always afford the best procedure because the concentration of the test material may vary considerably. Variations in material concentrations may be caused by several factors, including precipitation of the test substance, chemical interactions in the solution, deposition of test material on the container wall, uptake by the test organism, or interactions of test materials and excretion of the organisms. In the static test procedure, it is rather easy to prevent the concentration of test material from exceeding a maximum in the test cell but it is usually rather difficult to maintain the desired concentration. Because little equipment is usually required, the static bioassay is relatively easy to set up and it can be operated in a minimal area. The static test, in which no effluent is discharged, allows the accumulation of waste products which may themselves be toxic. As a result, static tests should be short-term tests. In static tests, it is advisable to utilize duplicates and even triplicates to insure test precision (Martin, 1973).

- Beginning the test

Static tests are begun either by:

- o adding toxicant to the test chambers 18 to 24 hours after the test organisms are added
- o or adding test organisms to the test chambers within 30 minutes after the toxicant is added to the dilution water

The first alternative allows the test organisms to partially acclimate to the test chambers and precludes loss of toxicant due to hydrolysis, sorption, or evaporation prior to exposure of the test organisms. The second alternative conserves dissolved oxygen and prevents the exposure of test organisms to the toxicant before it is evenly dispersed; this alternative must be used when the tests are conducted on aged solutions of a toxicant in dilution water (U.S. EPA, 1975a).

- Duration

Test organisms must be exposed for 96 hours in basic static test, for 48 to 96 hours in effluent static tests (U.S. EPA, 1975a).

3.3.2.3 Flow-through Bioassay--

Flow-through bioassay is more sophisticated than static bioassay and frequently involves a considerably greater amount of equipment. This methodology is the one currently being utilized by the majority of bioassay users, as it more closely approximates natural conditions. In this concept, the test organisms are held in a unit or cell into which continuous input of test solution, premixed in a dilution water, is metered. The operation requires maintenance of desired concentrations of test material; determination of residence time and the solution flow rate to the test cell must also be known. To accomplish the maintenance of a homogeneous concentration in the test unit requires the mixing of dosing solution of known concentration with a standaridized dilution water (Sprague, 1971).

In the flow-through bioassay, chemical tests should be run at intervals during the continuous flow test to assure that the test material concentrations are maintained in the desired range. Provisions must be made to feed and maintain the organisms during the test and excess food must be removed to limit the development of high bacterial populations. One of the major problems with the continuous test is that the dosing apparatus is sometimes difficult to control. The continuous flow-through test is particularly applicable where the wastes being tested are easily decomposed by bacterial action or when they are volatile or unstable and have a high biochemical oxygen demand (Martin, 1973).

- Flow-rate

The flow-rate must be at least 5 water volumes per 24 hours. The flow-rate through the test containers should not vary by more than 10% from any one test container to any other or from one time to another within a given test (U.S. EPA, 1976).

- Beginning the test

Flow-through tests are begun either by:

- placing the test organisms in the test chambers after the test solutions have been flowing through the test chambers long enough so that the toxicant concentrations are constant
- or activating the toxicant metering device in the toxicant delivery system several days after the test organisms were placed in test chambers that had dilution water flowing through them

The first alternative allows the investigator to study the behavior of the toxicant and the toxicant delivery system immediately prior to the beginning of the test, whereas the second alternative allows the test organisms to partially acclimate to the test chambers before the beginning of the test (U.S. EPA, 1975a).

- Duration

A test begins when the test organisms are first exposed to the toxicant. In the flow-through bioassay, all organisms must be exposed for at least 96 hours. When basic flow-through tests are conducted with large organisms (over 0.5 g each), it is usually desirable to determine the shape of the toxicity curve; i.e., LC50 or EC50 vs. time, throughout an 8-day exposure (U.S. EPA, 1975a).

3.3.2.4 Acute Bioassay--

Acute toxicity tests are generally used to determine the level of toxic agents that produce an adverse effect on a specified percentage of the test organisms in a short period of time. The most common acute toxicity test is the acute mortality test. Experimentally, 50% effect is the most reproducible measure of the toxicity of a toxic agent to a group of test organisms (U.S. EPA, 1975a).

- Experimental procedure

There are two procedures in current use:

- Approximate mortality times are recorded for most individual animals. The time taken to obtain 50 percent mortality is estimated for each test tank. The series of median lethal times is generally used to estimate an approximate threshold concentration for lethal effect (TL_m).
- Mortality is recorded only at 1, 2 and 4 days. The concentration lethal to half the test species at each time period is estimated (LC50)

The first procedure entails more complete observations and hence will also provide the answers yielded by the second procedure. However, the two procedures tend to yield similar results when exposure is for 4 days or more (Sprague, 1969).

- Required volume of test solution

This would probably depend on the size and shape of the holding tank to which the test animals were previously accustomed. Some recommendations about minimum depths and volumes are given by Doudoroff et al., (1951).

However, there does not seem to have been any investigation on exactly what size or shape of tanks are necessary to eliminate stressing the test species and affecting test results. It must be left in large part to the judgment of the investigator to provide enough water for a reasonable amount of free activity by the test animals.

EPA has proposed that for large fishes (over 0.5 g each) the test solution should be between 10 and 30 cm deep (U.S. EPA, 1975a). This problem may be avoided in a continuous flow test. Alabaster and Abram (1965) recommended that the supply of new test solution should be sufficient to maintain dissolved oxygen in the test tank. This also keeps toxicant and waste products within desirable limits. The extreme values which they mention for required amount of replacement solution are 0.5 and 10 liter per gram (for fish) per day (Sprague, 1969).

- Measuring response at each concentration

The reason for using a group of test animals in each test tank instead of one animal, is that individuals vary in resistance. Ever since Trevan (1927), it has been generally recognized that in bioassays, the least and most resistant individuals in a group show much greater variability in response than individuals near the median for the group. A good deal of accuracy may therefore be gained by measuring some average response rather than a minimum or maximum response, which might represent one animal in ten or might happen to represent only one animal in a thousand.

- Randomization

A serious systematic error could result from placing each successive batch of 10 captives (for fish) in a test tank in order of concentration. According to Gaddum (1953), distribution of animals by a process like dealing out a pack of cards (for example six tanks were to receive fish, the first fish which was caught would be placed in the first tank, the second into the second tank etc., the seventh into the first tank) still has a tendency to put more easily caught animals into certain concentrations. To avoid this, Finney (1964) suggests using random numbers. An improvement of this has been used in research by the U.S. Federal Water Pollution Control Administration and is hereby recommended as follows:

For six tanks, the first six fish to be caught from the holding tank are distributed one to each of the test tanks, in random order according to occurrence of the numerals 1 to 6 in a table of random numbers or by drawing numbered slips of paper; the seventh to twelfth fish are distributed one to each of the six tanks by the same process; this is continued until the tank is filled. In addition, test concentrations should also be assigned to the tanks by formal randomization to guard against any effect of position.

- Duration

To establish the time factor involved to produce an LC50 in acute bioassay several schemes have been used. Katz (1971) in one experiment (Table 3.3.3.7) showed that the 96-hour bioassay is unnecessarily long and does not yield anymore worthwhile information than does a 24- or 48-hour test.

TABLE 3.3.30 TIME FACTOR IN TOXICITY BIOASSAY TESTS[a] (Katz, 1971)

Waste ppm	pH	Fish Survival							
		24 hours		48 hours		72 hours		96 hours	
		Living	Dead	Living	Dead	Living	Dead	Living	Dead
		Replicate 1: July 21 - July 24[b]							
55.0	7.68	0	10	0	10	0	10	0	10
44.0	7.69	10	0	10	0	10	0	10	0
16.5	7.83	10	0	10	0	10	0	10	0
0.39 (control)	7.89	10	0	10	0	10	0	10	0
		Replicate 2: July 24 - July 29[c]							
49.0	7.69	2	8	2	8	2	8	2	8
45.0	7.69	4	6	4	6	4	6	4	6
43.0	7.69	9	1	9	1	9	1	9	1
0.13 (control)	7.89	10	0	10	0	10	0	10	0

[a] Test conditions: flowing water, 1.5 liters/hour

[b] TLm: 49 ppm waste

[c] TLm: 46 ppm waste

But according to Sprague (1969), the most popular exposure period is 4 days or 96 hours (Table 3.3.31).

TABLE 3.3.31 ESTIMATES OF TIME REQUIRED FOR CESSATION OF ACUTE LETHAL ACTION IN VARIOUS BIOASSAYS REPORTED IN THE LITERATURE (Sprague, 1969)

Toxicant	Species	Apparent Time of Lethal Threshold h=hour, d=day, w=week	Authors
Cyanide	Phoxinus	about 2 d	Wuhrmann, 1952
Cyanide	Trout	4 d or more	Herbert and Merkens, 1952
Ammonia	Trout	5 h	Lloyd, 1961b
Ammonia	4 freshwater fish	less than 4 d	Ball, 1967a
Ammonia	Phoxinus	about 2 d	Wuhrmann, 1952
Fluoride	Trout	about 7 d	Herbert and Shurben, 1964
Chlorine	Trout	more than 7 d	Merkens, 1958
High pH	Trout	more than 15 d	Jordan and Lloyd, 1964
Zinc	Minnow fry	1 d or less	Pickering and Vigor, 1965
Copper, zinc	Salmon	1 to 3 d	Sprague and Ramsay, 1965
Zinc	Zebrafish	1 to 6 d, various young stages	Skidmore, 1965

(continued)

TABLE 3.3.31 (Continued)

Toxicant	Species	Apparent Time of Lethal Threshold h=hour, d=day, w=week	Authors
Copper	Trout	2 to 4 d	Liepolt and Weber, 1958
Copper, zinc	Trout	4 d or less	Lloyd, 1960, 1961a
Heavy metals	Freshwater fish	2 d or less for about half of 59 cases; 4 d or longer for other half (static tests)	Pickering and Henderson, 1966a
Zinc	Minnow eggs	7 d or less	Pickering and Vigor, 1965
Zinc	4 freshwater fish	4 to 5 d	Ball, 1967b
Zinc	Bream	7 d or more	Ball, 1967b
Cadmium	Trout	7 d	Ball, 1967c
Eighteen metals	Stickleback	7 d or more in in each case	Doudoroff and Katz, 1953, from data of Jones, 1938 and 1939
Copper	Crayfish	10 to 15 d (delayed mortality)	Hubschman, 1967
Thallium	Perca	more than 14 d	Nehring, 1962
Various (6)	Tubificid worms	2 d or less	Marvan, 1963
Corrosion inhibitors	Trout	14 d or more	Herbert, 1965
ABS detergent	Bluegill	1 d or less (static tests)	Lemke and Mount, 1963
ABS detergent	11 freshwater fish	2 d or less (continuous flow)	Thatcher, 1966
Detergents	Trout	acute 1 d, subacute continued 12 w	Herbert et al., 1957
LAS detergent	5 freshwater fish	more than 4 d (continuous flow)	Thatcher and Santner, 1966
ABS, LAS detergents	Minnow eggs	9 d or more (continuous flow)	Pickering, 1966
Phenol	Trout	1 d or less (saline water)	Brown et al., 1967b
Phenol	4 freshwater fish	5 h to 1 d	Wuhrmann, 1952
Various phenolics	Trout	1 d	Brown et al., 1967a
Various petrochemicals	Freshwater fish	62 of 75 cases, 1 d or less; remainder 4 d or more (static tests) 1, 5 d	Pickering and Henderson, 1966b

(continued)

TABLE 3.3.31 (Continued)

Toxicant	Species	Apparent Time of Lethal Threshold h=hour, d=day w=week	Authors
DDT (acetone)	Salmon	1, 5 d	Alderdice and Worthington, 1959
DDT	Trout	acute 1, 5 d; subacute 2 w	Abram, 1967
Five insecticides	2 Stoneflies	30 d or more (several modes of action)	Jensen and Gaufin, 1966
Chlorinated hydrocarbon insecticides	4 freshwater fish	14 cases, 2 d or less; 8 cases, 4 d or more (static tests); continuous flow tests, 20 d or more	Henderson et al., 1959
Organophosphate	6 freshwater fish	41 cases, 2 d or less; 27 cases, 4 d or more (static tests)	Pickering et al., 1962
Various pesticides	Freshwater fish	25 cases, 2 d or less; 13 cases, 4 d or more (static tests)	Pickering and Henderson, 1966c
Sewage effluent	Trout	1 case, 8 h; 3 cases, about 3 d	Lloyd and Jordan, 1963
Pulp mill effluent	Salmon	about 12 d	Alderdice and Brett, 1957
Many pollutants	Various invertebrates, especially Daphnia	of 82 cases, 1 d or less, 26 cases; 1 to 3 d, 14 cases; 2 d or more, 13 cases; 4 d or more, 29 cases (static test)	Dowden and Bennet, 1965

Sprague (1969) realized that of 375 cases, 211 or 56% showed a lethal threshold in 4 days or less, while in the remaining 164 cases, lethality occurred beyond the 4th day. The overall distribution tended to substantiate that 4 days or 96 hours was a reasonable limit for occurrence of acutely lethal toxicity of most test substances. In view of this information, it would seem prudent to continue tests for 4 days as a rule. Tests could then be stopped if mortality had ceased and the toxicity curve showed a threshold.

- Methods

Examples of protocols for acute static bioassay with freshwater fish and daphnia and marine animals are given in the following pages:

EXAMPLE 1: ACUTE STATIC BIOASSAY WITH FRESHWATER FISH AND DAPHNIA

Purpose of Study

To determine the toxicity of chemicals to freshwater fish and daphnia.

Design of Experiment

- Test Animals

Fathead Minnow *Pimephales promelus*
Daphnia pulex (first instar stage)

- A series of test containers each with a different, but constant, concentration of toxicant will be used.
- At least 10 but preferably 20 organinisms should be used in each container for each treatment.
- For the minnow, the 96-hour median lethal concentration (96 hr-LC50) and for Daphnids, the 48-hr median effective concentration will be used.
- A series of controls will be used in which the water conditions, animal species and size will be the same as those used for each treatment group.
- The timing of the test and the collection of samples will be based on an understanding of the short and long-term operations and schedules of the discharge if possible.

Conduct of Experiment

- Select the test organisms.

QUALITY CONTROL -- Species must be readily available, hearty, and easy, convenient, and economical to maintain.

QUALITY CONTROL -- All minnows should be from the same year class, and weigh between 0.5 and 1.0 grams; the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than twice that of the shortest fish.

- Select dilution water.

QUALITY CONTROL -- A healthy test organism must survive in the dilution water for the duration of acclimation and testing without showing signs of stress, i.e., discoloration or unusual behavior.

- QUALITY CONTROL -- The test organism must survive and reproduce satisfactorily in the dilution water. A water in which Daphnids, who are more sensitive to many toxicants than most other freshwater aquatic animals, will survive and reproduce should be an acceptable dilution water for most tests with freshwater animals.

- At least two grab samples of effluent should be collected. The samples, whether liquid waste or sludge, should be stirred to a uniform consistency.

QUALITY CONTROL -- Conduct separate tests on each grab sample; more tests may be desirable if there are known sources of variability such as process changes.

QUALITY CONTROL -- The sample of the effluent must not be aerated or altered in any way except that it may be filtered through a sieve or screen with 2mm or larger holes.

QUALITY CONTROL -- Samples must be covered at all times, violent agitation must be avoided.

- Prepare stock solution or dilution of waste.

QUALITY CONTROL -- Add the same volume at all test levels.

QUALITY CONTROL -- The stability of the test substance in the stock solution should be determined.

- Place the test organisms in the test containers.

QUALITY CONTROL -- Stratified randomization or total randomization of the treatment is recommended.

QUALITY CONTROL -- True replicates with no water connection should be used.

QUALITY CONTROL -- The use of more animals and replication of treatment is desirable.

Observations and Results

- The final result should be expressed as concentration tolerated by the median or "average" test animal. A test is not acceptable if more than 10% of the organisms in any control die in a test determining LC50 or show effect in a test determining EC50.

- At a minimum, the number of dead or affected animals must be observed and recorded at 24-hour intervals. More observations, however, are desirable.

Termination

- At the end of test period, the bioassays are terminated and the LC50 or EC50 values are determined.

Records

- Any deviation from these methods should be recorded as well as the following specific information:

  The chemical characteristics of the the dilution water.

  Test organisms.

  Definition of the criterion used to determine the effect; abnormal behavior.

  Percentage of organisms that died or showed the effect in the control treatment.

  Duration.

  Statistical methods employed to interpret test results.

Report

- In addition to the final report, interim reports may be made available to the sponsor if required. The frequency of such reports will be determined prior to study initiation.

EXAMPLE 2. ACUTE STATIC BIOASSAY WITH MARINE ANIMALS

Purpose of Study

- Toxic Effect

Design of Experiment

- Test animals: juvenile sheepshead minnows (*Cyprinodon variegatus*);

adult grass shrimp (Palaemonetes pujio or P. vulgaris)

- 20 control animals and 20 test animals must be exposed to each concentration of test material.

- A control and 6 concentrations of effluent in a geometric series will be used.

- Concentration of test effluent that yields LC50 or EC50 values will be determined.

- The animals will be observed for 96 hours.

Conduct of the Experiment

- Select the test animals.
  QUALITY CONTROL -- The animals should be healthy and as uniform in size as possible.

  QUALITY CONTROL -- During holding, acclimation and testing, the animals must not be disturbed unnecessarily. When they must be handled, it must be as gently, carefully, and quickly as possible.

- Grab samples of effluent, whether liquid waste or sludge, should be stirred to a uniform consistency.
  QUALITY CONTROL -- Effluent samples may be filtered through a sieve or screen with 2mm or larger holes. The collection of samples should be based on an understanding of the short- and long-term operations and schedules of the discharges if possible.

- Check the salinity of undiluted effluent and add an appropriate amount of salts (Table 3.3.3) to yield a salinity of 10 parts per thousand as determined by a refractometer.

- Two range-finding tests should be performed: one with aeration and one without. To aerate, introduce clean air into the test effluent at the rate of 100 ± 15 bubbles per minute. Use effluent concentrations of 0.01, 0.1, 10 and 100 percent. If more than 50 percent of the animals die at 0.01 percent, conduct a new range-finding test at lower concentrations, such as 0.001 and 0.0001 percent.
  QUALITY CONTROL -- The stability of the effluent sample in the stock solution should be determined.

  QUALITY CONTROL -- Conduct a control test in 100% dilution water at the same time. The pH of the test media and control must be taken before and after the test.

- Determine the definitive test concentration from the results of the range-finding test. The concentration in each treatment must be at least 50 percent that of the next higher one. One treatment must kill more than 65% of the test animals and one treatment must kill less than 35%.

QUALITY CONTROL -- Stratified randomization or total randomization of the treatment is recommended.

QUALITY CONTROL -- True replicate with no water connection should be used.

QUALITY CONTROL -- The use of more test animals and replication of treatment is desirable.

QUALITY CONTROL -- A separate test should be conducted on at least two grab samples and more tests may be desirable if there are known sources of variability such as process changes.

Observations and Results

- Observe the animals frequently throughout the 96 hours and record the number of dead or affected animals for each 24-hour period. The final results will be expressed as concentration tolerated by the median or "average" animal. A test is not acceptable if more than 10% of the control animals die.

Termination

- At the end of the test period, the bioassays are terminated and the LC50 or EC50 values are determined.

Reocrds

- Records will be maintained on:

detailed description of the material tested

test animals

abnormalities such as erratic swimming, loss of reflex, discoloration, behavioral changes, excessive mucous production, hyperventilation, opaque eyes, curved spines, hemorrhaging, molting and cannibalism

percent of control animals that die or were affected in each test container during the test

duration

statistical method used for interpreting the result

Reports

In addition to the final report, interim reports may be available to the sponsor. The frequency of such reports will be determined prior to study initiation.

3.3.2.5 Chronic Bioassay--

Chronic bioassays are of primary value in determining "safe" levels of toxicants. All such tests involve exposures through the reproductive period of the life cycle and subsequent exposures of the eggs and young (Eaton, 1970). The use of the chronic test allows the test operator to better determine the most sensitive species or life stages to be assayed and on which organisms to base toxic limits (Martin, 1973).

Test procedures considered adequate are available for bluegill, fathead minnow, brook trout and Daphnia magna and procedures are being developed for several additional fish and invertebrate species. Various short-term tests have also been developed for use in conjunction with chronic tests (Eaton, 1970). A rather complete discussion of the chronic bioassay is presented by Sprague (1971).

The chronic tests differ from the acute tests in that they are an attempt to measure concentration harmful or safe to the system in a direct manner without using a lethal end point. The chronic test, as with the acute test, requires similar test operations. Usually continuous-flow test procedures are used and test dosages are maintained at levels below lethal concentration and the test is usually carried well beyond the conventional time period for the acute and/or static test (Martin, 1973).

Only this kind of exposure demonstrates the "safe" toxicant concentrations at which most life processes are protected. Usually the safe toxicant concentrations as determined by chronic bioassays are 10 to 100 times lower, and sometimes as much as 200 to 500 times lower than concentrations determined by acute bioassay using 50% mortality as an end point (Eaton, 1973).

- Experimental Procedure

Acute flow-through bioassays should be conducted prior to initiation of any chronic test. It is desirable for these tests to be on at least two different age classes (e.g., fry, juveniles or adults).

Concentrations selected for chronic toxicity experiments should be based on results of acute flow-through bioassays. Concentrations should be selected so that at least one will adversely affect some life stage of the test animal and one will not affect any stage.

Chronic bioassay usually includes exposure of animals to five or six toxicant concentrations along with a control; consecutive concentrations usually differ from one another by a factor of 2 or 3. Fish tests often start with 40 to 50 individuals per tank, and numbers are reduced at intervals for closer examination for toxicant effects and to adjust sex ratio so that only 6 to 20 remain at the time of spawning (Eaton, 1970). Fish chronic exposures routinely take about 10 months to a year to complete whereas _Daphnia magna_ are exposed for only 3 weeks, as they go through an entire life cycle in that time (Eaton, 1973). Use true duplicates for each level of toxic agent with no water connections between duplicate tanks (U.S. EPA, 1973). For _Daphnia magna_, true quadruplicates should be used (Biesinger, 1975).

- Water source

Freshwater: should be from a well or spring if at all possible, or alternatively from a surface water source. Only as a last resort should water from a chlorinated municipal water supply be used.

Saltwater: should be natural sea water with salinity greater than or equal to 15%.

Any proposed source must be analyzed for possible pollutants such as pesticides, PCB's and heavy metals. Special determinations should be made for those toxicants being investigated (U.S. EPA, 1976).

- Dosing apparatus

A number of apparatuses would be acceptable for this bioassay including those of Mount and Brungs, 1967; Hansen et al., 1971; Hansen et al., 1974b; or Schimmel et al., 1974 (U.S. EPA, 1976). The diluter should be checked daily, either directly or through measurement of toxicant concentrations. An automatically triggered emergency aeration and alarm system must be installed to alert staff in case of diluter, temperature control or water supply failure (U.S. EPA, 1973).

- Toxicant mixing chamber

A container to promote mixing of toxicant should be used between diluter and tanks for each concentration. Separate delivery tubes should run from this container to each duplicate tank. The whole system should be checked at least once every month to see that the intended amount of water is going to each duplicate tank or chamber (U.S. EPA, 1973).

- Spawning chamber

The spawning chamber should be small enough to be placed in an aquarium, but large enough to permit the female to avoid the aggressiveness of the male, and should be designed so eggs would sink through mesh bottom and fall on a surface for collection (Hansen and Parrish, 1976).

- Embryo and fry chamber

These chambers should be constructed to allow for adequate exchange of water and to insure that the proper quantity of material is entering the chambers. Care must be taken that each embryo and fry chamber receives an equal amount of the toxicant solution (U.S. EPA, 1976).

Exposure chamber, spawning chamber, hatching container, growth chamber and other equipment are varied to meet the needs of the different organisms used in the test (Rand et al., 1975).

- Photoperiod

Simulate the natural seasonal daylight and darkness periods with appropriate twilight periods. Make adjustments in photoperiods on the first and fifteenth of every test month (Rand et al., 1975). It may be desirable to control lights by a timing switch (Drummond and Dawson, 1970).

- Cleaning

All aquaria should be cleaned whenever material builds up. Aquaria should be brushed down and siphoned to remove accumulated material a minimum of 2 times weekly (U.S. EPA, 1973). Care should be exercised in cleaning to prevent loss or damage to the fry, juveniles, or adults (U.S. EPA, 1976).

- Disturbances

All test chambers should be shielded from excessive outside disturbances. Tanks should be shielded from all outside light sources that would interfere with the photoperiod (U.S. EPA, 1976).

- Test Animals

There are several criteria to be considered when choosing test organisms for a chronic bioassay:

> The test organisms should be able to reproduce readily in close confinement, producing large numbers of eggs;
>
> fertility as well as survival to adulthood should be high;
>
> the organisms should mature rapidly, yet be small enough at adult size to maintain large, statistically valid numbers of test organisms in the bioassay;
>
> the test organisms should be relatively sensitive to toxic pollutants (Schimmel & Hansen, 1974).

The test organisms should be obtained from the same source, either from wild population or suitable culture laboratory (U.S. EPA, 1976). To obtain a sufficient number of eggs to begin a chronic exposure, two methods may be employed:

natural spawning from laboratory stocks;

artificial inducement by injection of human gonadotrophic hormone and fertilization with sperm excised from males (Schimmel et al., 1974).

The former may be preferable.

- Food

Each batch of food should be checked for pesticides (DDT, Dieldrin, Endrin, etc.) and the kinds and amounts should be recorded (U.S. EPA, 1976).

- Disease

Disease outbreaks should be handled according to their nature with each aquarium being treated similarly even though disease is not evident in all aquaria. All treatments should be kept to the minimum and recorded as to type, amount, and frequency (U.S. EPA, 1976).

As mature adults begin courtship, separate pairs should be placed in individual spawning chambers in the aquaria. Pairs should be left in the chambers until a sufficient number of eggs have been collected to insure statistical comparisons of fecundity and fertility, and survival counts of embryo and fry can be made. All eggs should be removed at a fixed time of each day so that the adults are not overly disturbed and that disruption of activity will not occur. Daily records of spawning and egg numbers must be kept. Each pair should be observed daily for a minimum of 2 weeks. Impartially, 50 fertile eggs should be collected and incubated. If no spawning occurs at the highest concentration, eggs should be transferred from control spawns and incubated in the highest concentration to gain additional information. Survival of embryos, time required to hatch, hatching success, and survival of fry will be determined and recorded. Additional groups of 50 eggs from contaminated aquaria should be placed in control aquaria to determine if they contain chemicals toxic to embryo or fry.

Daily records on embryos and fry should be kept of mortalities and development of abnormalities. Termination of the chronic test is considered as the time when no spawning activity has occurred over a 2 week interval (U.S. EPA, 1976).

Data that must be reported for each tank of a chronic test are:

number and individual total length of normal and deformed test animals at 30 and 60 days; total length, weight and number of either sex, both normal and deformed, at end of test;

mortality during the test;

number of spawns and eggs;

hatchability;

fry survival, growth and deformities (U.S. EPA, 1973).

- Concentration of toxicant

A minimum of 5 concentrations of toxicant and a control, all duplicated, should be utilized in all chronic tests. Concentrations selected for chronic toxicity experiments should be based on results of acute flow-through bioassays. Concentrations should be selected so that at least one will adversely affect some life stage of the test animal and one will not affect any stage (U.S. EPA, 1976).

Concentrations of the toxicant should not vary by more than ± 10 to 15% from the selected test concentration because of uptake by the test organisms, absorption, precipitation and other causes (Rand et al., 1975).

Analyses should be made of the material itself, of the water during this test and of the test organisms (adult) at the conclusion of the test. At a minimum, water from each aquarium at the beginning and end of the test, and test animals from each aquarium (10 or more test animals each) at the end of the test, should be analyzed. It is highly desirable to chemically analyze additional samples of water and of test animals including, at each life stage, muscle tissue and gametes (U.S. EPA, 1976).

- Preparing a stock solution

If a toxicant cannot be introduced into the test water as is, a stock solution should be prepared by dissolving the toxicant in water or in an organic solvent. Acetone has been the most widely used solvent, but dimethylformamide (DMF) and triethylene glycol may be preferred in many cases. The use of solvents, surfactants, or other additives should be avoided whenever possible. If an additive is necessary, reagent grade or better should be used. The amount of an additive should be kept to a minimum, but the calculated concentration of a solvent to which any test organisms are exposed must never exceed one-thousandth of the 96-hour LC50 for test species under the test conditions and must never exceed 0.1 gram per liter of water. The calculated concentration of surfactant or other additive to which any test organisms are exposed must never exceed one-twentieth of the concentration of the toxicant and must never exceed 0.1 gram per liter of water. If any additive is used, two sets of controls must be used, one exposed to no additives and one exposed to the highest level of additives to which any other organisms in the test are exposed (U.S. EPA, 1973).

- o Measurements of other variables

Temperature must be recorded continuously. Dissolved oxygen must be measured in the tank daily, at least 5 days per week on an alternating basis, so that each tank is analyzed weekly for pH, alkalinity, hardness, acidity and conductance, or more often if necessary, to show the variability in the test water. At a minimum, the test water must be analyzed at the beginning and near the middle of the test for calcium, magnesium, sodium, potassium, chloride, sulfate, total solids, and total dissolved solids. Methods described in "Methods for Chemical Analysis of Water and Wastes" (U.S. EPA, 1974) should be used for those measurements. At a minimum, accuracy should be measured using the method of known additions for all analytical methods for toxicants.

If available, reference samples should be analyzed periodically for each analytical method (U.S. EPA, 1973).

- Methods

An example of a protocol for chronic flow-through bioassay with fish and aquatic invertebrates is given in the following pages.

EXAMPLE: CHRONIC FLOW-THROUGH BIOASSAY WITH FISH AND AQUATIC INVERTEBRATES

Purpose of the Study

- To determine the quantity of chemical that can be tolerated by fish and aquatic invertebrates.

Design of Experiment

- Start with 40 to 50 animals per tank. Use at least two different age classes.
- Expose animals in duplicate to five or six toxicant concentrations.
- Use a series of controls in which all test conditions will be similar to those of the experimental groups, except the toxicant will be absent from the test medium.
- Observe for 96 hours LC50.

Conduct of the Experiment

- Select test species.

QUALITY CONTROL -- The chosen species should be able to reproduce readily in close confinement, producing a large number of eggs. Fertility as well as survival to adulthood should be high.

- Fish tests should start with 40 to 50 individuals per tank and number should be reduced at intervals for closer examination for toxicant effects and to adjust sex ratios so that only 6 to 20 remain at the time of spawning.

- A chronic test should be used which includes exposure of animals in duplicate to 5 or 6 toxicant concentrations along with a control. Consecutive concentrations usually differ from one another by a factor of 2 or 3.

QUALITY CONTROL -- Stratified randomization or total randomization of the treatment is recommended.

QUALITY CONTROL -- True duplicate with no water connection between aquaria should be used.

QUALITY CONTROL -- The control should consist of the same water conditions and animals of the same species as are used in the remainder of the test. If any additive is present in any of the test chambers, an additive control is also required.

QUALITY CONTROL -- An acute flow-through bioassay should be conducted prior to initiation of any chronic test. It is desirable for these tests to be conducted with at least two different age classes.

- Use a proportional diluter for all long-term exposures.

QUALITY CONTROL -- The calibration of the toxicant delivery system should be checked daily before, during and after the test, either directly or through measurement of toxicant concentration.

QUALITY CONTROL -- If duplicate test containers are used, separate delivery tubes can be run from the mixing chambers to each duplicate.

QUALITY CONTROL -- Check at least once every month to see that the intended amounts of water are going to each duplicate tank or chamber.

QUALITY CONTROL -- A container to promote mixing of toxicant bearing water should be used between diluter and tank for each concentration.

Observations and Results

- Observe for mortalities and development of abnormalities.

- Obtain water quality criteria by multiplying the 96-hour LC50 of the most sensitive species tested by an arbitrary application factor.

Termination

Termination of the test is considered at the time when no spawning activity has occurred over a 2-week interval.

Records

Records will be maintained on:

- Detailed description of test material
- Test animals
- Percent of control animals that died or were affected in each test container during the test
- Daily records of spawning, egg numbers, fertility
- Mortalities and development of abnormalities of embryos and fry
- Number of spawns and eggs
- Hatchability
- Fry survival, growth, and deformities
- Duration
- Statistical methods used to interpret test results

3.3.2.6 Algal Bioassay--

The algal bioassay test is intended to identify algal growth-limiting nutrients, to biologically determine their availability, and to quantify the biological responses to changes in concentration.

These measurements are made in a uniform manner by inoculating test water with a selected algal test culture and determining algal growth at appropriate intervals.

- Species selection

In choosing species for bioassays, the following criteria are useful guides:

- o Whenever possible, indigenous species representing a diversity of phylogenetic types from the major seasonal succession should be studied.
- o The more sensitive species should be used.
- o Conditions of greatest vulnerabilities should be identified for the species selected.
- o Both test species and culture conditions should permit growth rates of 0.5 to 1.0 doublings per day under nonstress conditions (U.S. EPA, 1976).

- Culture conditions

The culture conditions for the test species generally should reflect their natural conditions.

- Marine algae

For temperate species, a temperature of 20±2°C and a light intensity of 2500 to 5000 lux on a 14-hour light and 10-hour dark cycle (14:10 cycle) are desirable.

For cold water species, a temperature of 8±2°C and 2500 to 5000 lux light intensity on 10:14 cycle is recommended (U.S. EPA, 1976).

- Freshwater algae

A temperature of 24±2°C and "cool white" fluorescent lamps giving at least 250 foot-candles (ftc) (2152 lux), preferably 400 ftc (4304 lux) are recommended (U.S. EPA, 1977).

- Selection of test water

  - Freshwater

Samples for the test may be:

surface samples from lakes and rivers,
wastewaters,
substances of concern that may ultimately reach surface waters,
any sample to which nutrients or other substances are added or from which they are removed.

  - Marine water

Sampling schedules should be arranged to take into account the tidal fluctuations, sampling preferably at high water, or at both high water and the following low water.

Transport samples to the laboratory at ice temperature. Temporary storage in the laboratory should occur under similar conditions. Each sample must be tested in triplicate (U.S. EPA, 1977).

- Concentration of spike

The volume of the spike should be as small as possible. The concentration of spikes will vary and must be matched to the waters being tested. Two considerations should be taken into account when selecting the concentrations of the spikes:

- The concentration should be kept small to minimize alterations of the sample, but at the same time it should be sufficiently large to yield a potentially measurable response.

- The concentration of spikes should be related to the fertility of the sample. To assess the effect of nutrient additions, they must be compared to an unspiked control of the test water.

In addition to spikes for the purpose of determining stimulatory or inhibitory effects on algal growth in est waters, it is sometimes necessary to check for the possibility that the test water contains some toxic materials which could influence results. To check for toxic materials, the test waters may be spiked with the elements in complete synthetic medium. If no increase in growth occurs, the presence of toxic materials is suspected (U.S. EPA, 1974b).

- Untreated controls

Control algal cultures must be grown in untreated medium (devoid of toxicant) at the time bioassays on liquid waste are being done (U.S. EPA, 1976).

- Test methods

Examples of protocols for unicelluar marine algal assay and fresh-water algal bottle test are given in the following pages.

EXAMPLE: UNICELLULAR MARINE ALGAL ASSAY

Purpose of Study

To determine biological response to changes in toxicant concentration.

Design of Experiment

- Select indigenous algal species or Skeletonema costatum.

- Use at least one control and five test concentration groups. The five concentrations must be in a geometric series and include concentrations that inhibit growth by approximately 65 and 35 percent.

- All tests should be performed in triplicate.

- Measure biomass once daily.

Conduct of Experiment

- Maintain algal stock cultures in artificial seawater medium of 10 parts per thousand salinity prepared from glass-distilled or deionized water.

QUALITY CONTROL -- Select the more sensitive algal species and the conditions of greatest vulnerabilities.

QUALITY CONTROL -- Test species and culture conditions should permit growth rates of 0.5 to 1.0 doublings/day under nonstress conditions.

QUALITY CONTROL -- Stock cultures must be manipulated according to standard microbiological techniques to insure a minimum of contamination by bacteria.

- Perform a toxicant concentration range finding test.

QUALITY CONTROL -- Perform in duplicate covering concentrations of 4 orders of magnitude.

QUALITY CONTROL -- If growth stimulation occurs, use 5 concentrations in a geometric series between a concentration without effect and 100 percent waste.

- When a range has been identified, dilutions of toxicant solutions should be prepared in distilled water or suitable solvent.

QUALITY CONTROL -- Stock solutions or dilutions of a waste should be prepared to assure that the same volume is added at all test levels. This addition should not exceed 1 ml per 50 ml of test medium with waste water.

- Control

QUALITY CONTROL -- Algal cultures must be grown in untreated medium at the time bioassays on liquid waste or sludge are being done.

Observations and Results

- Determine absorbance of the culture every day between days 3 and 12.

- Plot the average absorbance for each day using semilogarithmic paper and examine the shape of the curve.

QUALITY CONTROL -- Be careful in interpretation of data; some toxicants inhibit growth in the early stages of a test.

- Estimate final biomass on the 12th day by weighing an aliquot of each culture.

QUALITY CONTROL -- Use a vacuum less than 0.5 atmospheres to prevent cell breakage.

Termination

At the end of the 12-day test period, terminate the bioassays and determine the EC50.

Records

Record the following test data:

- EC50 at 12 days and other days of importance to be decided by the shape of the growth curve.

- The specific growth rate between days 3 and 12 and any other period depending upon the shape of the growth curve.

EXAMPLE 1: FRESHWATER ALGAL BOTTLE TEST

Purpose of the Study

- to identify algal growth-limiting nutrients;
- to determine biologically the availability of growth-limiting nutrients;
- to quantify the biological response to changes in concentrations of growth-limiting nutrients.

Design of Experiment

- Test algae:

  Selenastrum capricornutum Printz
  Microcystis aeruginosa Kutz. emend Elenkin
  (*Anacystis cyanea*) Drouet and Daily
  Anabaena flos-aquae (Lyngb.) De Brebisson
  Diatom - *cylotella* sp.
  - *Nitzschia* sp.

- The starting concentrations in the test water should be as follows:

  S. capricornutum 10^3 cells/ml
  M. aeruginosa and *A. flos-aquae* 50×10^3 cells/ml

- Measure biomass at least once daily

Conduct of the Experiment

- Select test species.
  QUALITY CONTROL -- Test species should be representative cross sections of types of algae found in waters of differing nutritional status.

- Collect water samples.
  QUALITY CONTROL -- Collect water samples in nonmetallic and autoclavable storage containers. Leave a minimum of airspace in transport container; keep in dark and at ice temperature.

  QUALITY CONTROL -- Do not reuse containers if toxic or nutrient contamination is suspected.

  QUALITY CONTROL -- Remove indigenous algae by membrane filtration (0.45 μ at 0.5 atmosphere or less) or autoclaving. Water can also be prefiltered through glass fiber filter.

  QUALITY CONTROL -- Duration of storage should be minimized.

- Select spikes of nitrogen, phosphorous, iron, sewage effluents, etc.
  QUALITY CONTROL -- Volume of the spikes should be as small as possible.

QUALITY CONTROL -- The concentration of the spike should be related to the fertility of the sample and should be kept small to minimize the alteration of the sample.

- The effect of nutrient additions must be compared to an unspiked control of test water.

QUALITY CONTROL -- Check for the possibility that the test water contains some toxic material which could influence results.

- Test each sample in triplicate.

QUALITY CONTROL -- For statistical purposes divide each into three aliquots before filtration and thereafter treat as separate samples.

Observations and Results

The fundamental measure used in the bottle test to describe algal growth is the amount of suspended solids (dry weight) produced; this is determined gravimetrically. Several different biomass indicators should be used whenever possible because biomass indicators may respond differently to any given nutrient-limiting condition.

Record

The following data should be recorded:

- the EC50 at 12 days and other days of importance to be decided upon by the shape of the growth curve;
- the specific growth rate between 3 and 12 days and any other period that should be reported depending upon the shape of the growth curve.

Report

In addition to the final report, interim reports may be made available to the sponsor if required. The frequency of such reports will be determined prior to study initiation.

- Results

Growth responses should be statistically analyzed and significant levels of differences reported. For most purposes a 95 percent significance level can be considered statistically significant. The EC50 can be estimated by interpolation by plotting the data on semilogarithmic coordinate paper with concentrations on the logarithmic axis and percentage growth in relation to the control on the arithmetic axis. Draw a straight line between two points on either side of the 50 percent growth value. The concentration at which the line crosses the 50 percent growth line is the EC50 value (U.S. EPA, 1977).

3.3.2.7 Community Studies --

Two examples of community studies follow:

- Purpose of study

To determine the effects of various types of alterations such as predator pressure, variability of the environment, and competition between species on species living together in a community.

- Experimental design

  - Periphyton community

To study the effect of germanium dioxide on a community, an experiment setup using periphyton was designed by Dickman (1969). Periphyton was chosen as representative of the community because (Rand et al., 1975):

- they are a very important food source for most forms of aquatic life that feed upon plants;
- they carry out the process of photosynthesis which is so important in the generation of oxygen needed by all organisms in order to carry out the metabolic processes;
- because of the large number of species, one will find many species present in almost all natural conditions;
- because as a group, they consist of many species that have populations composed of varying numbers of specimens;
- they are an excellent group to treat statistically in analyzing their reaction to varying ecological conditions.

- Conduct of Experiment

The basic procedure is to expose a set of slides with a suspected toxicant to the water column of a lake or stream where it would be possible for the periphyton to colonize it. A second set of slides identical to the first in every respect but lacking the suspected toxicant is suspended nearby for comparison. The species composition of the periphyton colonizing the two types of slides (treated and control) can then be compared at weekly intervals by harvesting some of the slides and allowing the remainder to continue to incubate. Significant differences in the species composition between the control and the treated slides can then be attributed to the presence of the substance which was impregnated on the slides.

In this study a chemical with a known toxic effect was chosen in order to test the proposed technique (in theroy, however, this technique should be applicable to any water-soluble substance). Germanium dioxide was chosen because its mode of action has already been demonstrated. In concentrations above 1.5 mg per liter the germanium dioxide suppresses silicon uptake and hence fission in diatoms.

- Periphyton sampling

The location on the slide at which a particular alga settles and grows is controlled largely by chance. Many of the algal species which appear to be rare in the water column may soon come to dominate the slides on which they settle. Five replicates should probably be a minimum under such circumstances.

One time-saving device is to record the data directly onto IBM forms so that they are ready for immediate punching. This also reduces the probability of error in copying the data from one form to another.

- Data analysis

The data from the enumeration of each slide were punched onto IBM computer cards. A program was written which:

- listed species counted per slide in the order of their relative abundance,
- calculated the diversity indices,
- calculated the mean abundance (density) and standard deviation of each species from the replicate slides,
- compared the above means for the treated and control slides at each time interval by means of Student's t-test.

- Disadvantages of this method

The major disadvantage in applying this technique to general use is that the concentration of the toxicant at the gel-water interphase is neither controllable nor known. The concentration of the toxicant to which the periphyton colonizing the slide are exposed will be a complex function of the following factors:

- the rate of water renewal at the gel-water interphase,
- the solubility of the compound being tested,
- the viscous flow characteristics and permeability of the gel.

Some of these factors are controllable. The acrylamide polymer gels have the advantage that they are not biodegradable as is agar.

- Advantages

The major advantage of this technique is its wide potential applicability. Whenever a pollutant is suspected, it could be impregnated in a gel and exposed to the periphyton in the same or similar area as that into which the potential toxicant would be released.

This method can be applied to marine as well as freshwater environments, flowing as well as stagnant waters. It can be employed at any time of the year and at nearly any possible location. In any case, such an approach obviates the necessity of extrapolating from over-simplified laboratory simulations or modeled environments. Furthermore, the necessary equipment is minimal and inexpensive. The results are easily quantifiable as a record of the effects of that particular compound (Dickman, 1969).

- Planktonic larvae community

A test procedure using a planktonic larvae community was designed by Hansen (1974) as follows:

- a planktonic larvae community is exposed to a test substance for

a relatively long period of time (usually 4 months);
- 10 aquaria and 10 replicates for each treatment are used (treatment includes control and contaminated apparatus);
- at the end of 4 months, the effects of contaminants on development of the community are determined by comparing the number, species and diversities of animals (Hansen, 1974).

o Planktonic larvae are selected because:

- plankton have long been used as indicators of water quality;
- some species flourish in highly eutrophic water while others are very sensitive to organic and/or chemical wastes;
- they have short life cycles; planktons respond quickly to environmental changes, and hence the standing crop and species composition indicate the quality of water mass in which they are found. As a group, they consist of many species that have populations composed of varying numbers of specimens. They are an excellent group to treat statistically in analyzing their reactions to varying ecological conditions (Rand et al., 1975).

• Test Substance

Polyethylene glycol 200 is recommended as solvent for most pesticides because this compound at 0.68-mg-per-liter, 2-ml-per-day concentrations, did not affect development of two species of crabs, and concentrations up to 1% (v/v) were not lethal to grass shrimp or sheepshead minnows in 96-hour static tests. The toxicity of 5 μg/liter of Aroclor 1254 to brown shrimp and pinfish was not increased by increasing the concentration of solvent up to 100 times (0.1 to 10.0 mg per liter). The same amount of solvent should be used in the control apparatus (Hansen, 1974).

For long-term studies, the concentrations of test substances shall be determined at the start of the study and samples shall be collected and analyzed periodically to verify concentrations (Hansen, 1974).

o Flow rate

The flow rate through each aquarium should be maintained at 200 ml per minute (Hansen, 1974).

o Concentration

The range 0.1, 1.0, 10.0 μg per liter of the toxicant seems adequate (Hansen, 1974).

• Termination

At the end of test period (4 months) the study is terminated and the index of species diversity as well as the percent occurrence of various species is determined (Hansen, 1974).

- Observations and results

Water is analyzed twice monthly and sediment is analyzed at the end of a 4-month period.

Modifications of the Shannon-Weaver method are used to assess effects of pollution on the natural community

$$H = -\sum_{i=1}^{s} p_i \log p_i$$

where p_i = proportion of the ith species in the collection
s = number of species.

Pooled data from each toxicant concentration and control are compared statistically using the χ^2 test for independent samples. Data from each of the 10 aquaria receiving one treatment are compared with data from 10 aquaria receiving a different treatment using the Mann-Whitney "U" test. Differences are considered real at alpha = 0.01.

3.3.2.8 Food-Chain Accumulation--

- Food-Chain Model

The buildup of certain substances, such as heavy metals, pesticides, etc., in the ecological food chain has been the subject of considerable study in recent years. Ecologists have attempted to analyze the flow of such material into various sectors of the ecosystem. To better understand the movement and transfer of toxicants throughout an estuarine trophic level, several food chain models or systems have been designed.

The model food chain is, in essence, a simple means to estimate, under controlled conditions, the movement of an organic synthetic chemical (i.e., a "foreign" molecule or xenobiotic) in certain representative trophic levels of a natural aquatic ecosystem.

A food-chain model should be inexpensive, simple to maintain, reproducible, ecologically relevant and able to produce clearly definable data (Johnson and Schoettger, 1975).

Thoman et al. (1974) have described a food-chain model of cadmium in western Lake Erie which is a mathematical model of the transfer of toxicants in the food chain. The purpose of the model is to:

- examine the structure of the buildup of potentially toxic substances in the food chain;
- determine what data would be required for a verification of the model;
- determine the utility and applicability of linear food-chain model in broadscale ecosystem planning;
- demonstrate the interfacing of nonlinear and linear modeling frameworks.

The model has proved useful in large-scale planning applications provided that additional data have been collected on the various trophic levels.

In a study of pesticide biodegradability, Metcalf et al. (1975) have proposed a laboratory model ecosystem with a terrestrial-aquatic interface and a seven-element food chain. The seven elements are:

Algae (Oedogonium cardiacum)
Snail (Physa)
Plankton
Water flea (Daphnia manga)
Mosquito pupae (Culex pupae)
Mosquito larvae (Culex larvae)
Mosquitofish (Gambusia affinis)

This food-chain model has been found very useful in estimating the potential environmental effects of DDT and other pesticides, particularly in regard to ecological magnification and biodegradability.

- Experimental Design

Generally the test procedure consists of:

- a series of test containers each with a different, but constant, concentration of toxicant;
- at least one control and three concentration groups;
- the number of animals per exposure ranging at least from 45 to 60;
- control consisting of the same water conditions, and animals of the same species and size which are used for the treatment groups;
- all tests performed in triplicate (Hamelink, 1976).

The use of ^{14}C compounds is recommended (Johnson and Schoettger, 1975).

- Test Animals

All test animals should be healthy and as uniform in size and age as possible. Test animals should be acclimated to laboratory test conditions for at least 10 days. Mortality of animals should not exceed 1% of the stock in the 48 hours immediately preceeding the test (U.S. EPA, 1975a).

Frequent disturbance and unnecessary handling should be particularly avoided because the environment of the animals has an immediate and profound influence on their respiration and metabolism.

The number of animals per exposure level is relatively large compared to most other toxicity tests. As a general rule, around 45 to 60 animals per tank is considered minimal. This quantity is necessary because three or more animals have to be sampled each period in order to accomodate the amount of individual variance encountered (Branson et al., 1975; Macek et al., 1975). About 12 to 15 sampling periods are usually required to establish the dynamics of both uptake and depuration (Hamelink, 1976).

A representative sample of test animals should be impartially distributed to the test containers by adding one or two test animals to each container, and then adding one or two more to each test container, and repeating the process until each test container has the desired number of animals in it. Alternatively, the animals can be assigned either by total randomization or by stratified randomization (U.S. EPA, 1975a).

For fish, only small fish must be used.

- Test Substance

The test sutstance should be technical grade. If a carrier or vehicle is used to dissolve or dilute the test substance, it should be chosen to possess as many of the following characteristics as possible:

- it should not interfere with absorption, distribution, metabolism or retention of the test substance;
- it should not alter the chemical properties of the test substance and not enhance, reduce or alter the toxic characteristics of the test substance;
- it should not affect the food and water consumption of the test organism;
- at the level used in the study, it should not produce physiological effects or have local or systemic toxicity (Anon., 1977).

If a solvent is used, two sets of controls, one with and one without solvent, should be used. The concentration of the toxicant under investigation should be relevant to the potential use of the information for registration or environmental impact statement reviews. The concentration of the toxicant used for a food-chain study should be selected on the basis of acute toxicity, recommended use rates, or information on probable concentrations likely to occur in aquatic ecosystems. Acute toxicity data or LC50 (lethal concentration) values probably represent the best information at present on which to base the selection of concentrations. Select the LC50 that represents the least tolerant member of the food-chain model. Concentrations of the toxicant used should not exceed the LC50. Concentrations between 1/10th and 1/1000th of the LC50, depending on the slope of the toxicity curve, should be used. However, other nonlethal concentrations are preferable if they can be estimated from an anticipated use rate from a concentration projected or measured in aquatic ecosystems (Johnson and Schoettger, 1975).

- Water Quality

Water should be uncontaminated and of constant quality and should meet the following qualifications:

- suspended solids <20 mg per liter;
- TOC or COD <10 mg per liter;
- unionized ammonia <20 μg per liter;
- residual chlorine <3 μg per liter;
- total organophosphorous pesticides <50 ng per liter;
- total organochlorine pesticides plus PCB's <50 ng per liter.

Water is considered to be constant in quality if the monthly ranges of the hardness, alkalinity, specific conductance TOC, or COD, and salinity are less than 10% of the respective averages and if the range of pH is less than 0.4 unit. Alternative freshwater should be obtained from an uncontaminated well or spring if possible; only as a last resort should dechlorinated water be used. If dechlorinated water is used, it must be shown that either first instar Daphnids can survive in it unfed for 48 hours or that residual chlorine measured below 3 mg per liter at the beginning of the test (U.S. EPA, 1975a).

- Test Duration

Test duration is determined by the time required to reach equilibrium. For a great majority of the pesticides studied by Macek et al. (1975), equilibrium was observed in a relatively short period of time (less than 3 weeks). However, in order to assess metabolism of the chemicals by fish and to be confident steady-state conditions have been reached, exposure periods ranging from 28 to 45 days are often employed.

- Size of Exposure Tank, Flow Rate and Turnover Time

Size of exposure tank and turnover time are determined by the total weight of the animals in each exposure level. Fish appear to require a minumum of 1 liter of water per gram per day (Branson et al., 1975; Macek et al., 1975; Reinert et al., 1974). There is a general tendency to increase the water turnover frequency as the average weight of the fish increases. This arises simply because it is generally easier to increase the flow rates than tank size (Hamelink, 1976).

The flow rate through the test chambers should not vary by more than 10% from any one test chamber to any other or from one time to another within the test.

- Sampling

All samples should be taken in replicates of 3 to 5 and expressed as mean values ± standard error; however, the chemical nature of the toxicant may necessitate a larger sample size. Data should not be utilized when mortality within the experimental group exceeds that in the control by 5% (Johnson and Schoettger, 1975).

- Chemical Analysis

Chemical analysis used in measuring uptake and degradation requires sensitivity sufficient to detect and quantify nanogram amounts. Radiolabeled compounds and radiometric assays consisting of liquid scintillation spectrometry and autoradiography or thin-layer chromatograms are recommended. The radioactive material, preferably ^{14}C-labeled, should occupy the most stable portion of the molecule. Efficiency of the radiometric system should be based on comparison with a spiked control (Johnson and Schoettger, 1975).

- Analysis of Results

Report all samples in terms of degradability of toxicant, percentage of degradation, and chromatographic identification of degradation products. Express all data as the mean ± standard error.

- Calculation

  - Plateau method

Calculate the mean and standard deviation of the concentration of chemicals in the water. A range of less than ±20% of the mean is desired. Alternatively, a time-weighted average can be determined by integration. Divide the concentration of chemical observed in the animals by the average concentration in the water. These values constitute the observed bioconcentration factor. Plot the observed bioconcentration factor versus time. If a plateau is observed, report the bioconcentration factor (BF) at or about the plateau region.

  - Kinetic methods

Plot the concentration observed in the animals versus time during exposure. Determine the slope for that initial period which can be observed to be linear to fit the uptake equation

$$C = a + K_1 t \qquad \text{Eq. 3.3.1}$$

where C = chemical concentration in animal (mg/liter)
a = y intercept
K_1 = uptake rate (mg/g/h)
t = time in hours

Plot the concentration observed in the animal during depuration on semilog paper versus time. If a straight line is apparent, determine the depuration equation

$$\ln C = a - K_2 t \qquad \text{Eq. 3.3.2}$$

where (C, a, and t are defined as above)
K_2 = clearance rate

When these two rates are equal, the equilibrium concentration is

$$0 = K_1 - K_2 Ce \qquad \text{Eq. 3.3.3}$$

where Ce = concentration of chemical in animals at equilibrium

By solving Eq. 3.3.3 for the Ce and dividing by the average concentration of chemical observed in the water (W), the projected BF at equilibrium is derived.

$$\frac{Ce}{W} = BF_e$$

3.3.2.9 Metabolic Bioassay--

Recurring pollution of natural waters from the manufacture and use of pesticides has accentuated the need for suitable monitoring methods. The determination of fish brain acetylcholinesterase (AchE) activity has been used for monitoring purposes. Gibson et al. (1969) exposed fish to organophosphate pesticides and showed that the mortality and recovery from organophosphorus poisoning are not necessarily related to the degree of AchE inhibition. Test specimens experiencing over 90% inhibition may fail to develop pronounced symptoms or organophosphorus poisoning and recover completely when removed to freshwater. They found great inhibition of AchE without death and death with little inhibition and therefore questioned the usefulness of AchE activity in the fish brains for monitoring. The confusing relationship between mortality and the degree of AchE inhibition jeopardized logical interpretation of data, i.e., the degree of AchE inhibition is not always related to the amount of toxicant present or to the length of exposure. Also, the cholinesterases are inhibited by more substances than any other group of enzymes.

However, recent laboratory and field studies have indicated that brain AchE inhibition in fishes is related to organophosphate insecticide poisoning. A specific level of brain AchE inhibition was shown to be related to deaths that occurred in a test population of sheepshead minnows (Cyprinodon variegatus) exposed to organophosphate insecticides in water under controlled static conditions in the laboratory (Coppage, 1972). Similar findings were made for AchE inhibitions in brains of cod (Gadus callarias) exposed in seawater in the laboratory to Paraoxon, a metabolite of the organophosphate insecticide parathion (Alsen et al., 1973).

Also, several field studies have shown that AchE inhibition in fish brain is correlated with water pollution or spraying with organophosphate pesticides in both fresh and estuarine water (Williams and Sova, 1966; Holland et al., 1967; Mayer and Walsh, 1970; Carter, 1971; Macek, et al., 1972).

A field study of three species of estuarine fishes showed that brain AchE inhibition was correlated with mosquito control operations with the organophosphate Malathion (Coppage and Duke, 1971).

Several methods have been used for the assay of cholinesterase. Most methods are based on the determination of the rate of disappearance of acetylcholine or the rate of formation of acetic or butyric acid from the hydrolysis of acetylcholine, acetyl-B-methylcholine or butyrylcholine (Witter, 1963).

There are two prerequisites for a satisfactory procedure:

- The rate of the reaction measured must be proportional to the amount of enzyme present. In other words, a straight line relationship must exist between enzyme concentration and enzyme activity.
- Enzyme measured under conditions of the assay must be a cholinesterase. Usually this is demonstrated by showing that low concentration of the

specific inhibitor eserine inhibits the hydrolysis of the substrate (acetylcholine).

Coppage (1971), in the study of the characteristics of brain AchE of sheephead minnow (*Cyprinodon variegatus*), has proposed a method for in vivo inhibition, utilizing the pH stat which overcomes many of the limitations and sources of error of other AchE assay methods.

In this test, 5 to 10 brains of adult fishes (40-70 mm total length) were pooled, weighed wet, homogenized in distilled water and diluted with distilled water to the desired tissue concentration. Acetylcholine iodide (Ach), acetyl-B-methylcholine iodide (Mech) and butyrylcholine iodide were used as ester substrates.

In vitro inhibitors were: guthion, phorate, diazinon and eserine sulfate.

For enzyme assay instrument, a Sargent recording of pH stat was used. Indicating the ability to meet prerequisites, the Figure 3.3.7 shows that the rate of hydrolysis of acetylcholine increased linearly with increasing amount of enzyme (brain homogenate). In addition, eserine completely inhibited hydrolysis of Ach at $1x10^{-4}$*M* concentration and inhibited hydrolysis by 81.5 percent at $1x10^{-6}$*M*, indicating hydrolysis is primarily caused by AchE (acetylcholine).

The inhibition values (Table 3.3.32) indicate that the presence of organophosphate pesticides can be detected by the pH stat brain AchE assay, but it is obvious that in vitro inhibition is not closely related to the toxicity of the compounds. Guthion is approximately 30 times as toxic as parathion but causes only about twice the inhibition.

This poor correlation between in vitro inhibition and in vivo toxicity can be explained by the fact that toxicity depends on in vivo AchE inhibition. Therefore, only in vivo inhibition could be a meaningful indicator of toxicity.

- Coppage's Proposed Techniques

Data from this study indicate that the following procedure is suitable for measuring normal and in vivo inhibited brain AchE with the automated pH stat: pool 5 to 10 brains from fish of similar size, weigh wet, homogenize in distilled water, and dilute with distilled water until tissue concentration is 5 mg per ml; mix 2 ml of diluted brain homogenate with 2 ml of 0.03*M* acetylcholine iodide in distilled water; titrate the liberated acetic acid with carbonate-free 0.01*N* NaOH; carry out the reaction at pH 7 and 22°C while passing nitrogen over the liquid to prevent absorption of atmospheric carbon dioxide. Calculate the micromoles of substrate hydrolyzed per unit of time from the number of micromoles of NaOH required to neutralize the liberated acetic acid per unit of time, and express AchE activity as micromoles of Ach hydrolyzed per hour per mg brain tissue.

For interpretation of in vivo inhibition, bioassay tests of fish in the laboratory should be made to determine the relationship of AchE inhibition

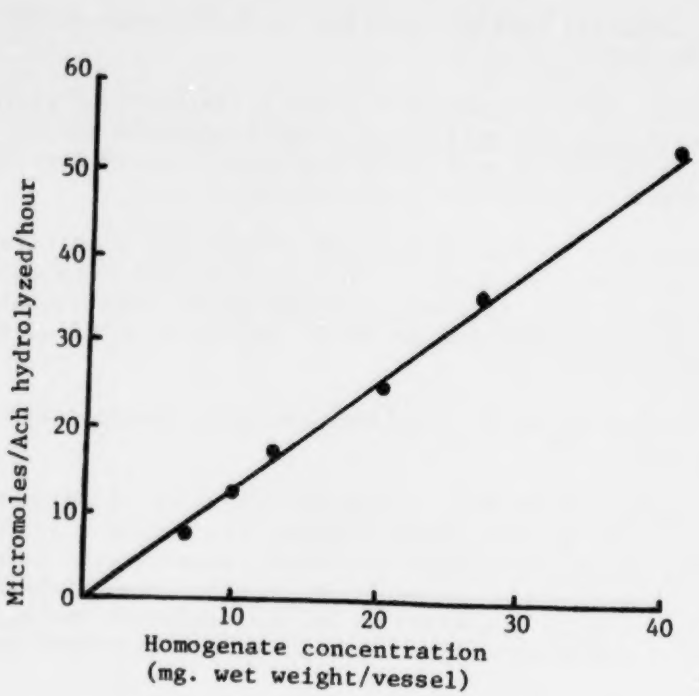

Figure 3.3.7 Hydrolysis of acetylcholine (15mM) by sheepshead minnow brain homogenate as a function of homogenate concentration.

TABLE 3.3.32 IN VITRO ORGANOPHOSPHATE PESTICIDE INHIBITION OF SHEEPSHEAD MINNOW BRAIN AchE COMPARED TO TOXICITY

Pesticide	Percent inhibition at $1\times10^{-4}\underline{M}$ concentration	48-hour LD50 (μg per liter of aquarium water)
Guthion	59.3	3.5
Phorate	31.5	9.0
Parathion	27.8	100.0
Diazinon	100.0	100.0

to pesticide concentration, length of exposure and death.

The assay method derived from studies in this work, when applied in tests comparing in vivo brain AchE inhibition and toxicity in sheepshead minnow, yields AchE activity measurements that correlate well with exposure and observed toxicity.

It is likely that a similar characterization and assay method would lead to improved correlation between brain AchE inhibition and observed toxicity in other fish.

The confusing relationship between mortality and degree of in vivo AchE inhibition reported by Gibson et al. (1964) is not evident in this test with the pH stat.

- Advantages of the method utilizing pH stat.
    - This method overcomes many of the limitations and sources of error of other AchE assay methods.
    - It does not utilize buffers.
    - It is rapid and simple to operate.
    - Rate curves are obtained by continuous recording of hydrolysis; also, pH, temperature, and enzyme and substrate concentration can be adjusted and maintained to permit studies of kinetics and optimum conditions.
    - It is not subjected to errors from color interference inherent in spectrophotometric methods.
    - It is not necessary to use substrates foreign to the enzyme, and small errors in substrate concentration would not significantly alter results as would be the case where residual Ach is measured (Coppage, 1971).

3.3.3 References

Abram, F. S. H. 1967. The definition and measurement of fish toxicity thresholds. In: Adv. Water Pollut. Res., Proc. 3rd Int. Conf. Munich, Germany, 1966. Vol. I., pp. 75-95. Pergamon Press, Oxford, England.

Abram, F. S. H. 1973. Apparatus for control of poison concentration in toxicity studies with fish. Water Res. 7: 1875-1879.

Alabaster, J. S., and F. S. H. Abram. 1965. Development and use of a direct method of evaluating toxicity to fish. In: Adv. Water Pollut. Res., Proc. 2nd Int. Conf. Tokyo, Japan. 1964. Vol. I. pp. 41-54. Pergamon Press, Oxford, England.

Alderdice, D. F., and J. R. Brett. 1957. Some effects of kraft mill effluent on young pacific salmon. J. Fish. Res. Board Can. 14: 783-795.

Alderdice, D. F., and M. E. Worthington. 1959. Toxicity of a DDT forest spray to young salmon. Can. Fish. Cult. 24: 41-48.

Alsen, C., et al. 1973. Characterization of cholinesterase of the cod (Gadus Callarias) and their in vivo inhibition by Paraoxon and Tabun. Arch. Toxikol 30: 263-275.

Anonymous. 1977. Toxic hazard evaluation of wildlife and aquatic organisms. EPA-OPP-Testing Program.

ASTM. 1974a. 1974. Standard method of test for evaluating acute toxicity of water to freshwater fishes. American Society for Testing and Materials, D. 1345-1359. pp. 212-221. Philadelphia, Pa.

ASTM. 1974b. Annual Book of ASTM Standards, Part 31. American Society for Testing and Materials, Philadelphia, Pa.

Ball, J. R. 1967a. The relative susceptibilities of some species of freshwater fish to poisons. I. Ammonia. Water Res. 1: 767-775.

Ball, J. R. 1967b. The relative susceptibilities of some species of freshwater fish to poisons. II. Zinc. Water Res. 1: 777-783.

Ball, J. R. 1967c. The toxicity of cadmium to rainbow trout. Water Res 1: 805-806.

Baudouin, M. F., and P. Scoppa. 1974. Acute toxicity of various metals to freshwater zooplankton. Bull. Environ. Contam. Toxicol. 12(6): 745.

Baudouin, M. F., and P. Scoppa. 1975. The determination of nucleic acids in freshwater plankton and its ecological implications. Freshwater Biol. Ass. Sci. Publ. 5:115.

Bengtsson, B. E. 1972. A simple principle for dosing apparatus in aquatic system. Arch. Hydrobiol. 70: 413-415.

Bennett, B. M. 1952. Estimation of LD50 by moving average. J. Hyg. 50(2): 157-164.

Biesinger, K. E. 1975. Tentative procedure for daphnia magna chronic tests in a flowing system. Fed. Regist. 40(123): 26903.

Bliss, C. I. 1937. The calculation of time-mortality curve. Ann. Appl. Biol. 24: 815-852.

Bliss, C. I. 1956. Confidence limits for measuring the precision of bioassays. Biometrics 12: 491-526.

Borror, A. C. 1963. Morphology and ecology of the benthic ciliated protozoa of Alligator Harbor, Florida. Arch. Protistenk. 106: 465.

Bourquin, A. W., et al. 1975. Inhibition of estuarine microorganisms by polychlorinated biphenyls. Develop. Ind. Microbiol. 16: 256-261.

Bovee, E. C. 1975. Effects of certain chemical pollutants on small aquatic animals. Kans. WRRI. Contrib. No. 157. Selected Water Resources Abstr. W75: 06981.

Branson, D. R., et al. 1975. Bioconcentration of 2,2',4,4'-tetrachlorobiphenyl in rainbow trout as measured by an accelerated test. Trans. Amer. Fish. Soc. 104: 785-792.

Brenniman, G., et al. 1976. A continuous-flow bioassay method to evaluate the effects of outboard motor exhausts and selected aromatic toxicants on fish. Water Res. 10: 165-170.

Brown, V. M., et al. 1967a. The effect of temperature on the acute toxicity of phenol to rainbow trout in hard water. Water Res. 1: 587-594.

Brown, V. M., et al. 1967b. The acute toxicity of phenol to rainbow trout in saline water. Water Res. 1: 683-685.

Canton, J. H., et al. 1975. Toxicity, accumulation and elimination studies of 2-hexachlorocyclohexane (α-HCH) with freshwater organisms of different trophic levels. Water Res. 9: 1163.

Carter, F. L. 1971. In vivo studies of brain acetylcholinesterase inhibition by organophosphate and carbamate insecticides in fish. Ph.D Dissertation, Louisiana State Univ., Baton Rouge, La.

Carter, J. W., and J. L. Cameron. 1973. Toxicity bioassay of heavy metals in water using Tetrahymena Pyriformis. Water Res. 7: 951.

Cline, T. F., and G. Post. 1972. Therapy of trout eggs infected with Sapolegnia. Prog. Fish-Cult. 34: 148-151.

Cooley, N. R., et al. 1972. Mirex and Aroclor 1254: Effect on and accumulation by Tetrahymena Pyriformis strain W. J. Protozool. 19: 636-638.

Cooley, N. R. 1973. Effects of pesticides on protozoa. In: Progress in Protozoology. Abstracts of papers presented at the Fourth International Congress of Protozoology, Clermont-Ferrand, France, September 1973. p. 91.

Cope, O. B. 1965. Sport fishery investigations. In: The Effects of Pesticides on Fish and Wildlife. U.S. Fish Wildl. Serv. Circ. No. 226: 51.

Cope, O. B. 1966. Contamination of the freshwater ecosystem by pesticides. J. Appl. Ecol. 3(Suppl): 33.

Coppage, D. L. 1971. Acetylcholinesterase with an automated pH stat for inhibition studies. Bull. Environ. Contam. Toxicol. 6(4): 304.

Coppage, D. L., and T. W. Duke. 1971. Effects of pesticides in estuaries along the gulf and southeast Atlantic coasts. In: Proceedings of the 2nd Gulf Coast Conference on Mosquito Suppression and Wildlife Management. C. H. Schmidt (ed.). National Mosquito Control Fish and Wildlife Management Coordinating Committee, Washington, D.C. pp. 24-31.

Coppage, D. L. 1972. Organophosphate pesticides: Specific level of brain AChE inhibition related to death in sheepshead minnow. Trans. Amer. Fish. Soc. 101: 534-536.

Corliss, J. O. 1970. The comparative systematics of species comprising the hymenostome ciliate genus *Tetrahymena*. J. Protozool. 17: 198.

Davis, H. S. 1954. Culture and diseases of game fishes. Univ. California Press, Berkeley, Calif.

DeFoe, D. L. 1975. Multichannel toxicant injection system for flow-through bioassays. J. Fish. Res. Board Can. 32: 544.

Dickman, M. 1969. A quantitative method for assessing the toxic effects of some water-soluble substances, based on changes in Periphyton community structure. Water Res. 3: 963-972.

Doudoroff, P., et al. 1951. Bioassay methods for toxicity to fish. Sewage Ind. Wastes 23(11): 1380.

Doudoroff, P., and M. Katz. 1953. Critical review of literature on the toxicity of industrial wastes and their components to fish. II. The metals, as salts. Sew. Ind. Wastes 25: 802-839.

Dowden, B. F., and H. J. Bennett. 1965. Toxicity of selected chemicals to certain animals. J. Water Pollut. Contr. Fed. 37: 1308.

Drummond, R. A., and W. F. Dawson. 1970. An inexpensive method for simulating diel patterns of lighting in the laboratory. Trans. Amer. Fish. Soc. 99(2): 434-435.

Eaton, J. G. 1970. Chronic malathion toxicity to the bluegill (Lepomis macrochirus Rafinesque). Water Res. 4: 673-684.

Eaton, J. G. 1973. Recent developments in the use of laboratory bioassays to determine "safe" levels of toxicants to fish. In: Bioassay Techniques and Environmental Chemistry. G. E. Glass (ed.). Ann Arbor Sci. Publ., Inc., Ann Arbor, Mich.

Elliott, A. M., et al. 1973. Biology of Tetrahymena. Dowden, Hutchinson and Ross, Inc., Stroudsburg, Pa.

Erickson, S. J., et al. 1970. A screening technique for estimating copper toxicity to estuarine phytoplankton. J. Water Pollut. Contr. Fed. 42: 270.

Finney, D. J. 1952. Probit Analysis. A statistical treatment of sigmoid response curve. 2nd Ed. Cambridge Univ. Press, London, England.

Finney, D. J. 1964. Statistical Method in Biological Assay. 2nd Ed. Hafner Publ. Company, New York, N.Y.

Freeman, R. A. 1971. A constant-flow delivery device for chronic bioassay. Trans. Amer. Fish. Soc. 100: 135-136.

Gaddum, J. H. 1953. Bioassays and mathematics. Pharmacol. Rev. 5: 87-134.

Gibson, J. R., et al. 1969. Sources of errors in the use of fish-brain acetylcholinesterase activity as a monitor for pollution. Bull. Environ. Contam. Toxicol. 4(1): 17.

Gillespie, D. M., et al. 1975. A kinetic model for static bioassay of insecticides. Water Res. 9: 817-819.

Glass, G. E. (ed.). 1973. Bioassay Techniques and Environmental Chemistry. Ann Arbor Sci. Publ., Inc., Ann Arbor, Mich. p. 139.

Granmo, A., and S. O. Kollberg. 1972. A new simple water flow system for accurate continuous-flow tests. Water Res. 6: 1597-1599.

Gregg, J. H., and A. G. Heath. 1975. A method for intermittent chlorine dosing in continuous-flow toxicity tests. Bull. Environ. Contam. Toxicol. 12(5): 588.

Gregory, W. W., et al. 1969. Accumulation of parathion and DDT by some algae and protozoa. J. Protozool. 16: 69-71.

Hamelink, J. 1976. Current bioconcentration test methods and theory. In: Aquatic Toxicology and Hazard Evaluation. Proceedings of a Symposium Sponsored by the ASTM Committee E-35 on Pesticides. F. L. Mayer and J. L. Hamelink (eds.). Memphis, Tenn. Oct. 25-26, 1976.

Hansen, D. J., et al. 1971. Chronic toxicity, uptake and retention of

Aroclor 1254 in two estuarine fishes. Bull. Environ. Contam. Toxicol. 6: 113-119.

Hansen, D. J. 1974. Effect on composition of developing estuarine animal communities in the laboratory. Mar. Sci. 18: 19-33.

Hansen, D. J., P. R. Parrish, and J. Forester. 1974a. Aroclor 1016: Toxicity to and uptake by estuarine animals. Environ. Res. 7: 363-373.

Hansen, D. J., et al. 1974b. Aroclor 1254 in eggs of sheepshead minnows. Effects of fertilization success and survival of embryo and fry. Proc. 28th Ann. Conf. Southeast. Ass. Game Fish. Comm. (in press).

Hansen, D. J., and P. R. Parrish. 1976. Suitability of sheepshead minnows (Cyprinodon variegatus) for life cycle toxicity tests. In: Aquatic Toxicology and Hazard Evaluation. Proceedings of a Symposium Sponsored by the ASTM Committee E-35 on Pesticides. F. L. Mayer and J. L. Hamelink (eds.). Memphis, Tenn. Oct. 25-26, 1976.

Harris, E. K. 1959. Confidence limits for the LD50 using the moving average-angle method. Biometrics 15: 424.

Henderson, C., et al. 1959. Relative toxicity of hydrocarbon insecticides to four species of fish. Trans. Amer. Fish. Soc. 88: 23-32.

Herbert, D. V. M., and J. C. Merkens. 1952. Toxicity of potassium cyanide to trout. J. Exp. Biol. 29: 632-649.

Herbert, D. V. M., et al. 1957. Toxicity of synthetic detergents to rainbow trout. Water Waste Treat. 6: 394-397.

Herbert, D. V. M., and D. S. Shurben. 1964. The toxicity of fluoride to rainbow trout. Water Waste Treat. 10: 141-142.

Herbert, D. V. M. 1965. Pollution and Fisheries. Ecol. Ind. Soc. 5th Symp. British Ecol. Soc. 173-195. Blackwell Scientific Publications, Oxford and Edinburgh, England.

Hoffman, G. L., and F. P. Meyer. 1974. Parasites of freshwater fishes. TFH Publications, Inc., Neptune City, N.J.

Holland, H. T., et al. 1967. Use of fish-brain acetylcholinesterase to monitor pollution by organophosphorus pesticides. Bull. Environ. Contam. Toxicol. 2: 156-162.

Hollister, T. A., and G. Walsh. 1973. Differential responses by marine phytoplankton to herbicides: Oxygen evolution. Bull. Environ. Contam. Toxicol. 9: 291-295.

Hubschman, J. H. 1967. Effects of copper on the crayfish. I. Acute toxicity. Crustaceana 12: 33-42.

Jensen, K. 1975. Hatching rate as bioassay. Proposal for a standard technique. Bull. Environ. Contam. Toxicol. 14(5): 562.

Jensen, L. D., and A. R. Gaufin. 1966. Acute and long-term effects of organic insecticides on two species of stonefly naiads. J. Water Pollut. Contr. Fed. 38: 1273-1286.

Johannes, R. E. 1965. Influence of marine protozoa on nutrient generation. Limnol. Oceanogr. 10: 434-442.

Johnson, B. T., and R. A. Schoettger. 1975. A biological model for estimating the uptake, transfer and degradation of xenobiotics in an aquatic food chain. Fed. Regist. 40(12): 26906-26909.

Jones, J. R. E. 1938. The relative toxicity of salts of lead, zinc and copper to the stickleback (Gasterosteus Aculeatus) and the effect of calcium on the toxicity of lead and zinc salts. J. Exp. Biol. 15: 394.

Jones, J. R. E. 1939. The relation between the electrolytic solution pressures of the metals and their toxicity to the stickleback. J. Exp. Biol. 16: 425-437.

Jordan, D. H. M., and R. Lloyd. 1964. The resistance of rainbow trout (Salmo Gairdnerii Richardson) and roach (Rutilus rutilus) (L) to alkaline solutions. Int. J. Air Water Pollut. 8: 405-409.

Katz, M. 1971. Toxicity bioassay technique using aquatic organisms. In: Water and Water Pollution Handbook, Vol. 2. L. L. Ciaccio (ed.). Marcel Dekker, Inc., New York, N.Y. p. 763.

Keil, J. E., et al. 1972. DDT and polychlorinated biphenyl (Aroclor 1242) effects of uptake on E. choli growth. Water Res. 6: 837-841.

Kester, D. R., I. W. Duedall, D. N. Connors, and K. M. Pytkowicz. 1967. Preparation of artificial seawater. Limnol. Oceanogr. 12: 176-179.

LaRoche, G. R., R. Eisler, and C. M. Tarzwell. 1970. Bioassay procedures to oil and oil dispersant toxicity evaluation. J. Water Pollut. Contr. Fed. 42: 1982-1989.

Lemke, A. E., and D. L. Mount. 1963. Some effect of alkyl benzene sulfonate on the bluegill. Trans. Amer. Fish. Soc. 92: 372-378.

Lenon, R. E. 1967. Selected strains of fish as bioassay animals. Progr. Fish-Cult. 29(3): 129-132.

Lichatowich, J. A., et al. 1973. Development of methodology and apparatus for the bioassay of oil. In: Proceedings of Joint Conference on Prevention and Control of Oil Spills. American Petroleum Institute, U.S. Environmental Protection Agency, and U.S. Coast Guard. Washington, D.C. pp. 659-666.

Liepolt, R., and E. Weber. 1958. Die Giftwirkung von Kupfersulfat auf Wasserorganismen. Wasser Abwasser, p. 355.

Litchfield, J. T. 1949. A method for rapid graphic solution of time-percent effect curves. J. Pharmacol. Exp. Ther. 97: 394.

Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96: 99-113.

Litchfield, J. T., and F. Wilcoxon. 1953. The reliability of graphic estimates of relative potency from dose-percent effect curves. J. Pharmacol. Exp. Ther. 108: 18-25.

Lloyd, R. 1960. The toxicity of zinc sulfate to rainbow trout. Ann. Appl. Biol. 48: 84-94.

Lloyd, R. 1961a. The toxicity of mixtures of zinc and copper sulfates to rainbow trout. Ann. Appl. Biol. 49: 535-538.

Lloyd, R. 1961b. The toxicity of ammonia to rainbow trout. Water Waste Treat. 8: 278-279.

Lloyd, R., and D. H. M. Jordan. 1963. Predicted and observed toxicities of several sewage effluents to rainbow trout. J. Inst. Sew. Purif., Pt. 2: 167-173.

Lowe, J. I. 1964. Chronic exposure of spot to sublethal concentrations of toxaphene in seawater. Trans. Amer. Fish. Soc. 92: 396-399.

Macek, K. J., et al 1972. Toxicity of the insecticide dursban to fish and aquatic invertebrates in ponds. Trans. Amer. Fish. Soc. 101: 420-427.

Macek, K. J., et al. 1975. Structure-activity correlations in studies of toxicity and bioconcentration with aquatic organisms. Published by the International Joint Commission, 100 Ouellette Ave., Windsor, Ontario, Canada N9A GT3. Chapter 7, March 1975, pp. 119-142.

Marking, L. L., and V. K. Dawson. 1973. Toxicity of quinaldine sulfate to fish. Invest. Fish. Control No. 48. U.S. Fish Wildl. Serv., Washington, D.C.

Martin, D. M. 1973. Freshwater Laboratory Bioassays. A Tool in Environmental Decisions No. 3. Contributions from the Department of Limnology, Academy of Natural Sciences of Philadelphia, Pa. p. 26.

Marvan, P. 1963. Zur Methodik der Auswertung von Toxizitätstesten. Sbornik Vysoke'skoly Chemicko-technologicke. V. Praze. Technologie Vody 7(1): 271-313.

Mayer, F. L., and D. F. Walsh. 1970. Multiple exposures of bluegill and aquatic invertebrate to abate. U.S. Bur. Sport Fish. Wildl. Res. Publ. 106: 16.

McKee, J. E., and H. W. Wolf (eds.). 1963. Water Quality Criteria, 2nd Edition, 548 pp. State Water Quality Control Board, Pub. 3-A, Sacramento, Calif.

McLachlan, J. 1960. The Culture of *Dunaliella Tertiolecta Butcher*. A euryhaline organism. Can. J. Microbiol. 6: 367-379.

Merkens, J. C. 1958. Studies on the toxicity of chlorine and chloramine to the rainbow trout. Water Waste Treat. 7: 150-151.

Metcalf, R., et al. 1975. Laboratory model ecosystem studies of the degradation and fate of radiolabeled tri-, tetra- and pentachlorobiphenyl compared with DDE. Arch. Environ. Contam. Toxicol. 3: 151-165.

Mount, D. I., and W. A. Brungs. 1967. A simplified dosing apparatus for fish toxicological studies. Water Res. 1(1): 21-29.

Mount, D. I. 1968. Test animals for water quality. Amer. Fish. Soc. Newsletter 12(54): 3-6.

Nehring, D. 1962. Untersuchunger über die toxikologische Wirkung von Thalliumionen auf Fische und Fischnahrtiere. Z. Fisch 11: 557-561.

Nehring, R. B. 1976. Aquatic insects as biological monitors of heavy metal pollution. Bull. Environ. Contam. Toxicol. 15(2): 147-154.

Ozburn, G. W., and D. Smith Alasdair. 1976. A mechanical toxicant injector for flow-through toxicity tests. Lakehead University, Thunder Bay, Ontario, Canada.

Patrick, R. 1964. Tentative method of test for evaluating inhibitory toxicity of industrial waste waters. American Society for Testing and Materials. ASTM Designation D.2037.

Payne, A. G. 1975. Response of the three test algae of the algal assay procedure: bottle test. Water Res. 9: 437-445.

Pickering, Q. H., et al. 1962. The toxicity of organic phosphorus insecticides to different species of warm water fishes. Trans. Amer. Fish. Soc. 19: 175-184.

Pickering, Q. H., and W. N. Vigor. 1965. The acute toxicity of zinc to eggs and fry of the fathead minnow. Progr. Fish-Cult. 27: 153-157.

Pickering, Q. H. 1966. Acute toxicity of alkyl benzene sulfonate and linear alkylate sulfonate to the eggs of the fathead minnow. Int. J. Air Water Pollut. 10: 385-391.

Pickering, Q. H., and C. Henderson. 1966a. The acute toxicity of some heavy metals to different species of warm water fishes. Int. J. Air Water Pollut. 10: 453-463.

Pickering, Q. H., and C. Henderson. 1966b. Acute toxicity of some important petrochemicals to fish. J. Water Pollut. Contr. Fed. 38: 1419.

Pickering, Q. H., and C. Henderson. 1966c. The acute toxicity of some pesticides to fish. Ohio J. Sci. 66: 508-513.

Provasoli, L. 1963. Organic regulation of phytoplankton fertility. In: The Sea, Vol. II. M. N. Hill, L. Lasko, C. Shaw, and E. Wirhowski (eds.). John Wiley and Sons, New York, N.Y. pp. 165-219.

Rand, M. C., A. E. Greenberg, and M. J. Taras. 1975. Standard Methods for the Examination of Water and Wastewater. 14th Edition. American Public Health Association, American Water Works Association, and Water Pollution Control Federation, Washington, D.C.

Rehwoldt, R., 1973. The acute toxicity of some heavy metal ions toward benthic organisms. Bull. Environ. Contam. Toxicol. 10(5): 291.

Reichenbach-Klinke, H., and E. Elkan. 1972. The principal diseases of lower vertebrates. Book 1. Diseases of Fishes. ISBN 0-87666-042-1, PS-205.

Reinert, R. E., et al. 1974. Proceedings 17th Conference Great Lakes Research, published by the International Association for Great Lakes Research. 1974. pp. 52-58.

Roberts, D. 1975. The effects of pesticides on Byssus formation in the common mussel, Mytilus Edulis. Environ. Pollut. 8: 241.

Sanders, H. O. 1970. Toxicities of some herbicides to six species of freshwater crustaceans. J. Water Pollut. Contr. Fed. 42(8): 1544.

Scheier, A. 1973. Personal communication. Limnology Department, Academy of Natural Sciences of Philadelphia. Philadelphia, Pa.

Schimmel, S. C., et al. 1974. Effects of Aroclor 1254 on laboratory-reared embryo and fry of sheepshead minnow. Trans. Amer. Fish. Soc. 103: 582-586.

Schimmel, S. C., and D. J. Hansen. 1974. Sheepshead minnow (Cyprinodon variegatus): An estuarine fish suitable for chronic (entire life-cycle) bioassays. Proc. 28th Ann. Conf. Southeast. Ass. Game Fish. Comm.

Shepard, M. P. 1955. Resistance and tolerance of young speckled trout (Salvelinus Fontinalis) to oxygen lack, with special reference to low oxygen acclimation. J. Fish. Res. Board Can. 12: 387-446.

Shumway, D. L., and J. R. Palensky. 1973. Impairment of the flavor of fish by water pollutants. U.S. Environmental Protection Agency, EPA-R$_3$-73-010, Washington, D.C.

Skidmore, J. F. 1965. Resistance to zinc sulfate of the zebrafish at different phases of its life history. Ann. Appl. Biol. 56: 47-53.

Snieszko, S. F. (ed.). 1970. A Symposium on Diseases of Fishes and Shellfishes. Spec. Publ. 5. American Fisheries Socity, Washington, D.C. p. 526.

Specht, D. T., and C. Miller. 1973. Development of a Standard Marine Algal Assay Procedure for Nutrient Assessment. In: Proceedings of Seminar on Methodology for Monitoring the Marine Environment. Seattle, Wash. October 1973.

Sprague, J. B., and B. A. Ramsay. 1965. Lethal levels of mixed copper-zinc solutions for juvenile salmon. J. Fish. Res. Board Can. 22: 425-432.

Sprague, J. B. 1969. Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. Water Res. 3: 793-821.

Sprague, J. B. 1971. Measurement of pollutant toxicity to fish. III. Sublethal effects and safe concentration. Water Res. 5: 245-266.

Stephan, C. E. 1973. Chemistry and fish toxicology. In: Bioassay Techniques and Environmental Chemistry. G. E. Glass (ed.). Ann Arbor Sci. Publ., Inc., Ann Arbor, Mich.

Stephan, C. E. 1976. Methods for calculating an LC50. In: Aquatic Toxicology and Hazard Evaluation. Proceedings of a Symposium sponsored by the ASTM Committee E-35 on Pesticides. F. L. Mayer and J. L. Hamelink (eds.). Memphis, Tenn. Oct. 25-26, 1976.

Sturm, R. N., and A. G. Payne. 1973. Environmental testing of trisodium nitrilotriacetate (NTA). Bioassays for aquatic safety and algae stimulation. In: Bioassay Techniques and Environmental Chemistry. G. E. Glass (ed.). Ann Arbor Sci. Publ., Inc., Ann Arbor, Mich. pp. 403-424.

Tattersfield, F., and H. M. Morris. 1924. An apparatus for testing the toxic values of contact insecticides under controlled conditions. Bull. Entomol. Res. 14: 223-233.

Thatcher, T. O. 1966. The comparative lethal toxicity of a mixture of hard ABS detergent products to 11 species of fishes. Int. J. Air Water Pollut. 10: 585-590.

Thatcher, T. O., and J. F. Santner. 1966. Acute toxicity of LAS to various fish species. Proc. 21st Ind. Waste Conf. Purdue Univ. Engin. Exten. Ser. No. 121: 996-1002.

Thoman, R., et al. 1974. A food chain model of cadmium in Western Lake Erie. Water Res. 8: 841-849.

Trevan, J. W. 1927. The error of determination of toxicity. Proc. Roy. Soc. B. 101: 483-514.

Trudgill, P. W., et al. 1971. Effect of organochlorine insecticides on bacterial growth, respiration and viability. J. Gen. Microbiol. 69: 1-3.

U.S. EPA. 1973. Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents. C. I. Weber (ed.). U.S. Environmental Protection Agency, EPA-670/4-73-001, Cincinnati, Ohio.

U.S. EPA. 1974a. Methods for Chemical Analysis of Water and Wastes. U.S. Environmental Protection Agency, EPA-625/6-74-003, Cincinnati, Ohio.

U.S. EPA. 1974b. Marine Algal Assay Procedure: Bottle Test. U.S. Environmental Protection Agency, PB-239709/9BA, Corvallis, Ore.

U.S. EPA. 1975a. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. U.S. Environmental Protection Agency, EPA-660/3-75-009, Corvallis, Ore.

U.S. EPA, Region II. 1975b. Quality Assurance Manual - Biology Section. U.S. Environmental Protection Agency, Region II, Edison, N.J.

U.S. EPA. 1976. Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Environmental Research Laboratory, EPA-600/9-76-010, Gulf Breeze, Fla.

U.S. EPA. 1977. IERL-RTP-Procedures Manual: Level 1. Biological Tests for Pilot Studies. U.S. Environmental Protection Agency, Research Triangle Park, N.C.

Van Duijn, C., Jr. 1973. Diseases of Fishes. 3rd Ed. Charles C. Thomas, Publ. Springfield, Ill. p. 309.

Vigers, G. A., and A. W. Maynard. 1977. The residual oxygen bioassay. A rapid procedure to predict effluent toxicity to rainbow trout. Water Res. 11: 343-346.

Walker, G., et al., 1975. Barnacles: Possible indicators of zinc pollution. Mar. Biol. 30: 57; Selected Water. Res. Abs. W75-11611.

Warnick, S. L., and H. L. Bell. 1969. The acute toxicity of some heavy metals to different species of aquatic insects. J. Water Pollut. Contr. Fed. 41(2): 280.

Weil, C. S. 1952. Tables for convenient calculation of median effective dose (LD50 or EC50) and instruction in their use. Biometrics 8: 249-263.

Williams, A. K., and R. C. Sova. 1966. Acetylcholinesterase levels in brains of fishes from polluted waters. Bull. Environ. Contam. Toxicol. 1: 198-204.

Witter, R. F. 1963. Measurement of blood cholinesterase. Arch. Environ. Health 6: 537.

Wuhrmann, K. 1952. Sur quelques principles de la toxicologie du poisson (Concerning some principles of the toxicology of fish). Bull. Cent. Belge Etude Doc. Eaux 15: p. 49 (Fish. Res. Board Can., Translation series No. 243).

Zaroogian, G. E., et al. 1969. Formulation of an artificial seawater media suitable for oyster larvae development. Amer. Zool. 9: 1141.

Zillioux, E. J., et al. 1973. Using artemia to assay oil dispersant toxicities. J. Water Pollut. Contr. Fed. 45: 2389-2396.

3.4 MICROBIOLOGIC ASSAY

The elements of a program to insure validity and integrity of experimental results in microbiologic work are essentially the same as those for other areas of biology: personnel, supervision, sampling, procurement and acceptance specifications, instrument checks and calibration, experimental design, standard test methods, controls (positive and negative), statistical analysis of data, and proficiency testing.

Competent, dedicated, industrious personnel are essential to the success of any program. Although there is no substitute for competence, continuing education, workshops, and on-the-job training can do much to raise the level of performance (Russell et al., 1969; Prier, 1973; Bartlett et al., 1968; Lott, 1973). The dedicated worker can be counted on for the extra time and effort that oft-times spells the difference between success and failure in a project. Indolent employees, on the other hand, may resort to short cuts and improvisions that can lead to erroneous results. Automated systems, where applicable, can eliminate, to a large degree, human errors due to such factors as eye fatigue. Automation has been a great boon in analytical chemistry and hematology. However, much remains to be accomplished in this area of microbiology technology (Heden and Illeni, 1974; Kuzel and Kavanagh, 1971; Kavanagh, 1974; Rippere and Arret, 1972).

Supervision must be professional and thorough. This important aspect of the program cannot be delegated to technical personnel or relegated to the status of a casual walk-through inspection from time to time by a busy administrator with many other duties constantly demanding attention. The proper supervision of a successful quality control program for a large laboratory is a major administrative task. The supervisor must make certain that all elements of the program are in operation at all times. A perfunctory examination of logbooks and test results will not insure this; the supervisor must be in the operation.

The other elements of the quality control program vary according to the nature of the project and will be discussed specifically in the following sections.

3.4.1 Microorganisms - Diagnostic Environmental Microbiology

Quality control in microbiology received a great impetus with the passage of the Federal Clinical Laboratories Improvement Act (CLIA) of 1967 which established minimum standards for clinical laboratories engaged in interstate commerce in the U.S.A. (PHS, 1968). A wealth of information and experience is now available for organizations launching programs in this area (Russell et al., 1969; Prier et al., 1973; Vera, 1971; Halstead et al., 1971; Glasser et al., 1971).

3.4.1.1 Sampling--

Environmental samples for the isolation and identification of pathogenic microorganisms must be representative, of sufficient size, and properly preserved so that viability of the agents isolated is preserved.

Water sampling methods for sanitary bacteriology have been developed and refined over a period of many years and are described in detail in Standard Methods for Examination of Water and Wastewater, 14th Edition, 1975 (Rand et al., 1975). Samples for judging water quality according to the 1975 USEPA Drinking Water Standards should be collected in sterile bottles that have been properly cleaned and rinsed with distilled water. A dechlorinating agent should be added unless the sample is collected in broth for direct plating. Sodium thiosulfate is usually added for dechlorination prior to sterilization in an amount sufficient to yield a final concentration of 100 mg/l of sample. Water samples high in copper or zinc or wastewater samples high in heavy metals should be collected in bottles which also contain a chelating agent such as ethylenediaminetetraacetic acid in an amount to give a final concentration of 372 mg/l. Individual samples should be taken at representative stations over the complete distribution system. The minimum number of samples to be collected each month is determined by the size of the population dependent upon the supply. Distribution system taps should be opened for 2 to 3 minutes, or long enough to empty the service line, before collecting the sample.

Other samples for bacteriologic examination of water should be representative and collected in a manner that precludes contamination. Well water should be hand-pumped for about 5 minutes before collecting the sample. The critical factor in collecting samples from a stream, lake, reservoir, spring, or shallow well is that the sample be representative of the body of water sampled. Samples from a stream may be taken at one-quarter, one-half, and three-quarters the width at various sites. The sample bottle should be held near the base and plunged neck downward to the desired depth and then turned slightly upward with mouth toward the current. Flow patterns and other hydrologic factors in streams as well as the tendency of motile organisms to gather where light, temperature, oxygen, nutrients, and/or flow are favorable, present difficulties in collecting a representative sample. The use of a standard Kemmerer Sampler for collecting multiple discrete samples at various depths and the continuous automatic-type sampler for collecting samples proportional to the flow pattern of the stream should merit consideration (Bicking, 1976). Samples collected from a boat should always be taken from the upstream side of the craft. Samples from moderate depths may be taken by attaching a weight to the base of the sample bottle. Deep sampling devices such as the ZoBell J-Z Sampler may be used for collecting samples at various depths from a lake or reservoir. The device consists of a 350-ml bottle with glass and rubber tubing equipped with a cable and a messenger. The messenger is released when the bottle is at the desired depth and breaks the glass tubing at a point weakened by a file mark and the sample is sucked in under a partial vacuum created at the time of assembly. Although impounded waters do not present as many hydrologic problems with respect to sampling, stratification and other factors make multiple sampling imperative to be truly representative. Bottom sediment samples may be collected with a Von Donsul and Geldreich sampler consisting of a stainless steel frame and a sterile plastic bag equipped with a nylon cord which closes the bag when the sampler penetrates the sediment.

Water samples should be tested as soon as possible after collection to insure valid results. Samples that cannot be analyzed within one hour after collection should be refrigerated at a temperature below 10°C. The maximum time between sampling and transportation of refrigerated samples to the laboratory should be no longer than 6 hours. Samples handled in this manner should be refrigerated on receipt at the laboratory and processed within 2 hours.

The major groups of pathogenic microorganisms that may be present in surface and groundwaters in the U.S.A. are Salmonella, Shigella, pathogenic Escherichia coli, Leptospira, and enteric viruses. Vibrio cholerae should also be considered in view of present-day widespread world travel. Standard methods of sampling for these groups of microorganisms in water have not been developed at this time. In general, however, some method of concentrating the sample must be employed since these organisms are present in much smaller numbers than the coliforms which are the index of pollution in sanitary bacteriology. Three techniques are recommended in Standard Methods (Rand et al., 1975).

- Swabs are prepared from a 216-cm length of 23-cm wide cheesecloth folded five times at 36-cm intervals. This provides a rectangle 23 cm wide on the folds by 36 cm long on the open edges, and six layers thick. Cut this lengthwise to within 10 cm of the head into 4.5-cm wide strips or streamers (four cuts making five streamers). Tightly wrap the uncut end with 16-gauge wire. For sampling, the swab is placed slightly below the surface of the stream for 3 to 5 days and traps microorganisms and other particulates. Water expressed from the swab, and pieces of the swab itself, are placed in enrichment media for analysis. Gauze pads of the same thickness may be substituted for the cheesecloth swabs.

- Diatomaceous earth ("Cellite", etc.) packed over an absorbent pad in a membrane filter holder may be used for concentrating microorganisms. At least two liters of sample should be drawn through the filter mass by vacuum. Representative samples of the filter "plug" are then sampled for analysis.

- Commercial membrane filters, 0.45-μm pore diameter, are satisfactory for concentrating pathogenic microorganisms in samples with low turbidity. Several liters of sample should be used.

Human enteric viruses excreted with the feces into domestic sewage constitute a special problem in water management. Viral particles in the center of clumps, covered by debris, or otherwise protected, may escape inactivation and eventually find their way to fully virulent form into a community water supply. Although there are only six viruses known to be shed in large numbers from the human intestinal tract - poliovirus, echovirus, coxsackie virus, reovirus, adenovirus, and infectious hepatitus virus - each occurs in varying numbers of different antigenic types so that today well over 100 different human enteric viral serotypes are recognized. Outbreaks of waterborne viral disease continue to be reported both here and abroad and there is considerable concern about larger and more serious outbreaks in the future (Craun et al., 1976). Fortunately, viruses are unable to multiply outside living cells and, unlike bacteria, do not increase in numbers in the water supply. This creates a special problem in water virology, however, since large volumes of sample (400-1900 liters) must be processed through filters or adsorbents to insure isolation of sufficient infectious units for viral identification. Great progress has been made in this area during the past decade, largely through the efforts of Metcalf (1961), Cliver (1967), Berg et al. (1971), Jakubowski et al. (1974), Hill et al. (1976), Wallis and Melnick (1967), and Wallis et al. (1972). A tentative microporus filter technique for enteric virus concentration in finished waters has been included in the latest (14th) edition of Standard Methods (Figure 3.4.1.).

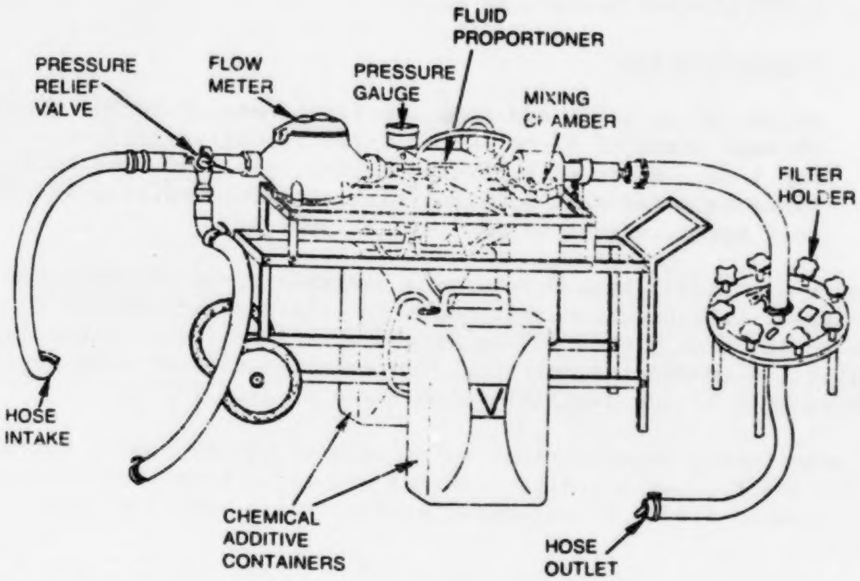
FLUID PROPORTIONER
PRESSURE RELIEF VALVE
FLOW METER
PRESSURE GAUGE
MIXING CHAMBER
FILTER HOLDER
HOSE INTAKE
CHEMICAL ADDITIVE CONTAINERS
HOSE OUTLET

Figure 3.4.1. Diagrammatic view of the virus-concentrator apparatus. Ancillary component parts are shown mounted on a two-wheeled dolly constructed of angle iron. Note: Use stainless steel fittings for all connections. (Rand et al., 1975)

The main features of the virus-concentrator apparatus are:

- Virus adsorbent

  8-µm + 1.2-µm stack of 293-mm cellulose nitrate membrane filters
  or
  5-µm + 1-µm stack of 267-mm epoxy-fiberglass-asbestos discs
  or
  3 epoxy-fiberglass tubes, 8 µm, 24.5 x 63.5 mm in parallel

- Sample treatment (continuous)

  pH 3.5

  Sodium thiosulfate (1:100 final conc.)

- Sample size

  400 to 2000 liters (to detect 1 to 2 infectious units/400 l)

- Flow rate

  4 to 10 liters per minute

- Elution

  0.05M glycine buffer, pH 11.5

- Reconstitution

  Adjust pH to 3.5. Add $AlCl_3$ to final conc. 0.0005M. Filter through stack of 47-mm AA^3Cox M-780 fiberglass filters 5 μm and 1 μm. Elute with glycine buffer, pH 11.5, into buffered Hanks balanced salt solution with nutrient broth or 20% fetal calf serum, adjust to pH 7.4.

Wallis et al. (1972) have developed a portable virus concentrator for isolating viruses from highly turbid tapwater. Yarn-wound clarifying filters are used in conjunction with a 293-mm size membrane filter or fiberglass textile filter. A commercial unit that concentrates viruses from water and elutes them as well is now available also (Rand et al., 1975).

Since subclinical enteric viral infections are quite common during the summer months the following quality control practices have been advocated by Akin and Jakubowski (1976) to safeguard against false positive results in water analysis.

- Personnel directly involved in sample collecting and handling should routinely have throat and rectal swabs collected. They should be processed if a virus-positive water sample is found
- Aseptic technique and a closed system should be used for sample collecting and processing
- When samples are to be stored prior to testing, they should be placed in ultralow temperature freezers that contain no other type of virus sample
- Samples should be processed in isolation facilities where no other type of virus sample is handled
- Multiple barriers to air contamination should exist, i.e., separate isolation facility, laminar flow hoods, etc.
- All isolates must be confirmed as being viral

A new instrument for large-volume sampling of water supplies for microorganisms was announced recently by the Bacterial and Parasitic Diseases

Section, Epidemiology Branch, Field Studies Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio. The portable instrument is adaptable for collection of bacteria, viruses, or Giardia cysts.

A flow diagram illustrating instrument operation for collection of viruses is given in Figure 3.4.2. Slight modifications in flow are necessary for collecting bacteria or Giardia cysts.

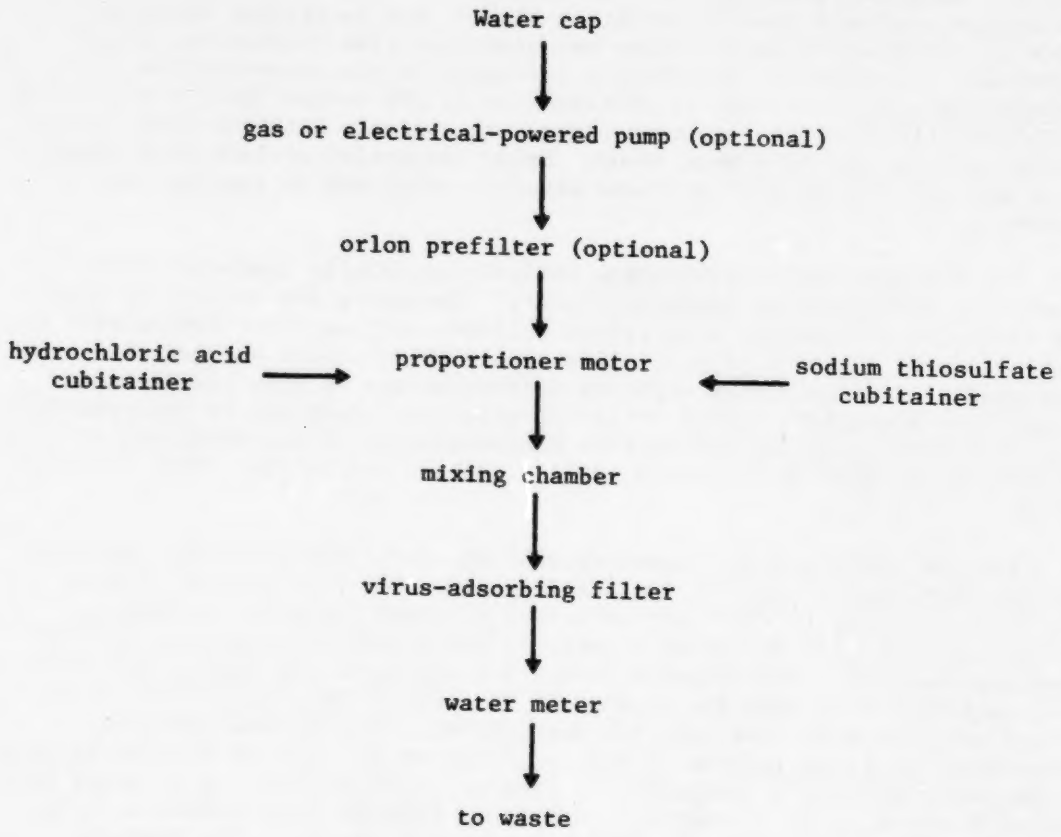
Water cap
gas or electrical-powered pump (optional)
orlon prefilter (optional)
hydrochloric acid cubitainer
proportioner motor
sodium thiosulfate cubitainer
mixing chamber
virus-adsorbing filter
water meter
to waste

Figure 3.4.2 Equipment configuration for virus sample collection.

Bacteria are collected on five Balston 0.3-μm filters, using one filter for each 75 liters of water. Giardia cysts are collected on a 25 cm long yarn-wound orlon pre-filter over a 24-hour period from a domestic water supply at the maximum flow rate obtainable. The flow rate is reduced to 4 liters per minute if the water is turbid. Viruses are concentrated in essentially the same manner as described in Standard Methods for the Examination of Water and Wastewater, 14th Edition (Rand et al., 1975).

The atmosphere contains all main groups of microorganisms including viruses. Mold spores are particularly prevalent and include species pathogenic for man, domestic animals, and plants. Coccidioidomycosis, histoplasmosis, cryptococcosis, aspergillosis, blastomycosis, and nocardiosis are typical human pulmonary diseases caused by pathogenic fungi found in the air. Coccidioidomycosis spores are so prevalent in the air in the San Joaquin Valley in California that the disease there is referred to as San Joaquin Valley Fever. Viruses have not been found in significant numbers in the outdoor air although there is some evidence for the airborne spread of both smallpox and foot-and-mouth disease (Davies, 1971; Jacobson and Morris, 1976). Soil and bodies of water are the main sources of bacteria in the outdoor air but sewage treatment plants, rendering plants, and facilities where solid waste is shredded for incineration may also give rise to airborne microorganisms. For example, significant increases in the concentration of Escherichia coli in the air at distances up to 800 meters (half a mile) from trickling filter sewage treatment plants, in contrast with the level in the upwind control air, have been found. Spray irrigation of land with chlorinated sewage effluent also produces aerosols which may be carried long distances.

Air sampling for microbiologic analysis is usually conducted with impactors, impingers, or membrane filters. Impactors are devices in which the airstream is directed onto sticky surfaces such as petri dishes with an agar medium or coated plates or slides where the microbes are trapped (impacted). Impingers trap airborne microorganisms as they are blown or sucked into a nutrient liquid or buffer solution. Membrane or alginate filters will filter out and concentrate microorganisms in the airstream in proportion to the pore size (Davies, 1971; Jacobson and Morris, 1976; Giever, 1976).

The two main types of impactors used in recent years for air sampling are the Slit Sampler and the Cascade Impactor. The Slit Sampler (Figure 3.4.3) is a device in which air is sucked or blown through a narrow slit orifice onto an agar medium in a rotating petri dish where the microorganisms are impacted. Particulates from a 3 cubic meter air sample are impacted onto each agar dish when the instrument is operated at a flow rate of 50 liters per minute for one hour (Goddard, 1976). The Cascade Impactor (Figure 3.4.4) is an instrument with a series of air jets of decreasing size in series to achieve a gradation in size of particles passing or being impacted from one stage to another. Impactors such as Petri dishes with an agar nutrient medium are positioned beneath each air jet. The Andersen Cascade Sampler (Figure 3.4.5) is regarded by some authorities as the best device today for air sampling of bacteria. With this instrument, air is drawn or blown through a circular opening and then through a series of six circular plates with 400 holes each onto the surface of agar media in underlying Petri dishes where the entrained particles are impacted. The plates have progressively smaller holes so that the largest particles are impacted on the first dish and the smallest at the sixth stage. Air is sampled at the rate of 28.3 liters per minute and retention is reported to be as great as 100% for single bacteria cells, although there is some loss on walls and plates. Impactors made up of seven or more units followed by a filter are also available (Figure 3.4.6). Particulates as small as 0.24 μm can be

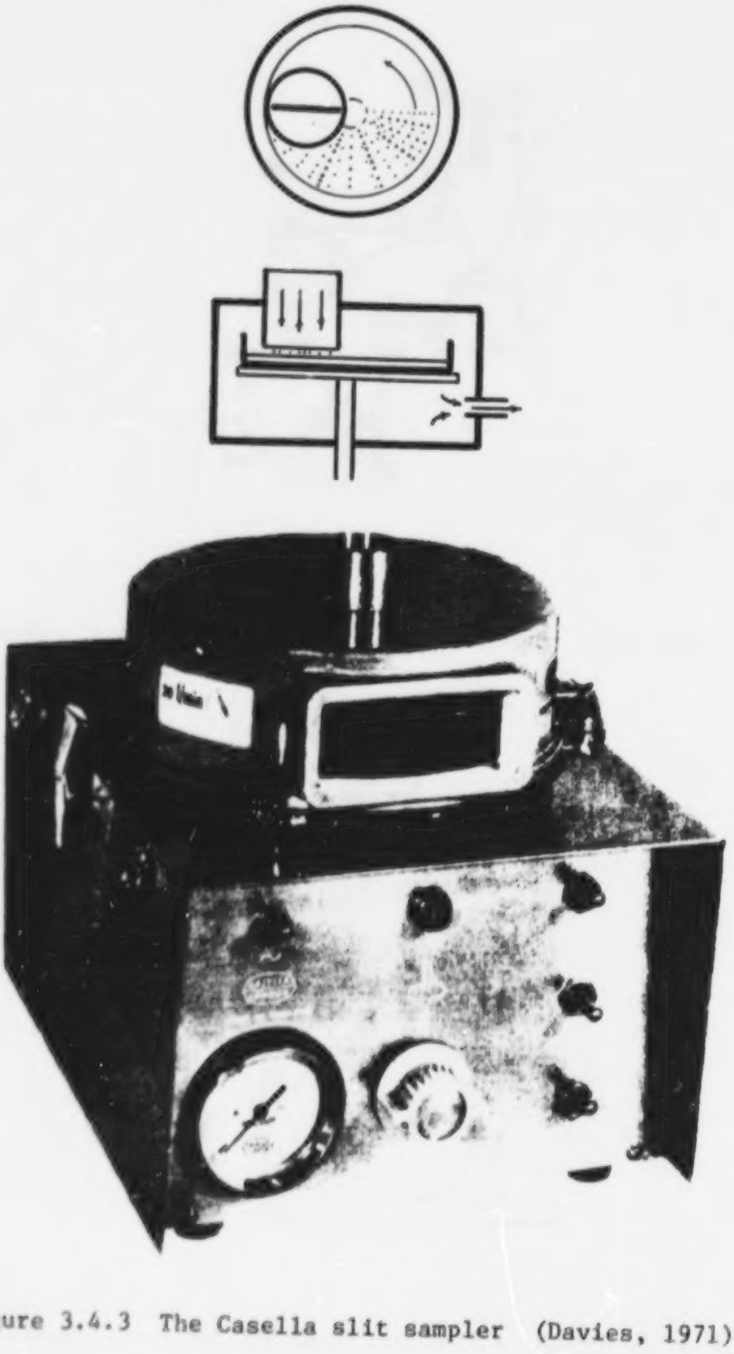

Figure 3.4.3 The Casella slit sampler (Davies, 1971)

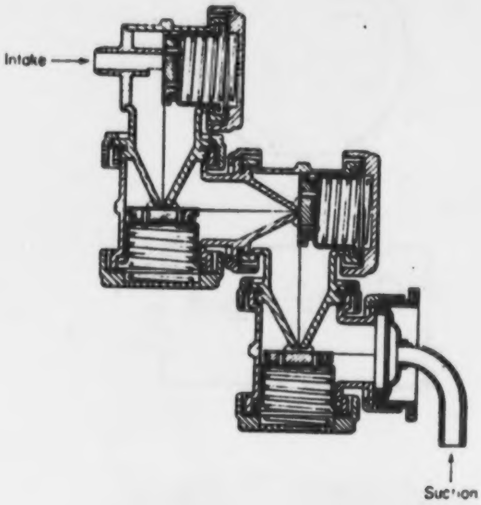
Intake
Suction

Figure 3.4.4 Sectional elevation of the Cascade Impactor (Davies, 1971)

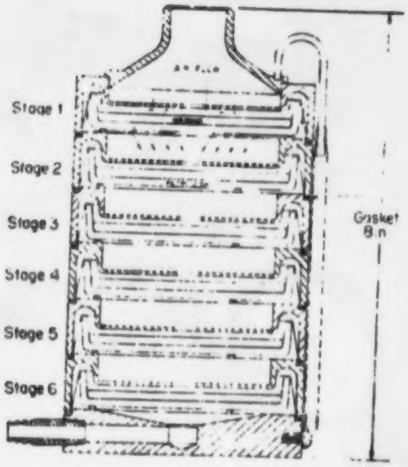
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
Stage 6
Gasket
8 in

Figure 3.4.5 Sectional elevation, Andersen Sampler (Davies, 1971)

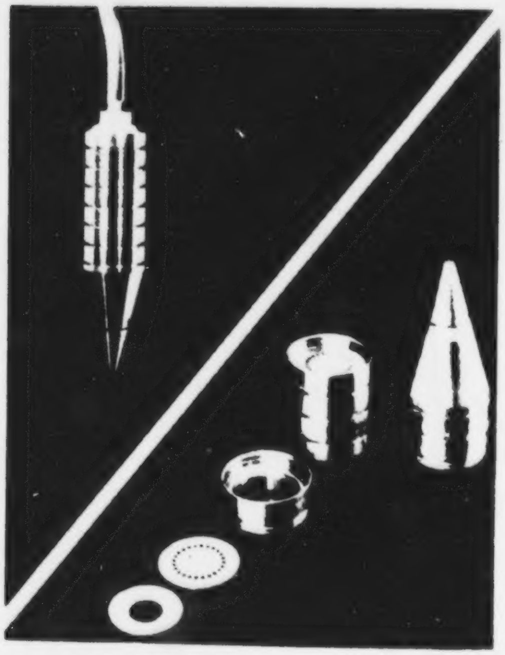

Figure 3.4.6 Inertial in-stack cascade impactor (Courtesy of Meteorology Research Inc.) (Giever, 1976)

collected in the last stage of typical modern commercial cascade impactors.

Impingers have several important advantages over impactors in isolating microorganisms from air:

- Aliquots of the liquid sample can be subcultured in a variety of enrichment broths and selective media which increases the possibility of isolating all of the various types of microbes trapped
- Loss of delicate, fastidious, and slow-growing species which often occur on crowded Petri dish cultures is avoided
- Viruses can easily be separated from bacteria and other types of microorganisms for isolation and identification

The Porton impinger (Figure 3.4.7), although simple in design and operation, is regarded as highly efficient for collecting single bacterial cells as small as 0.5 to 1μm in diameter. A slow flow rate (11 liters per minute) and foaming are its main deficiencies. The Multi-stage Liquid Impinger (Figure 3.4.8), consisting of three chambers separated by sintered glass discs of graded pore size has obvious advantages over the single-cell impingers. Discs and walls are continuously wetted by the collecting medium and foaming is reduced to a minimum even at high flow rates. This instrument

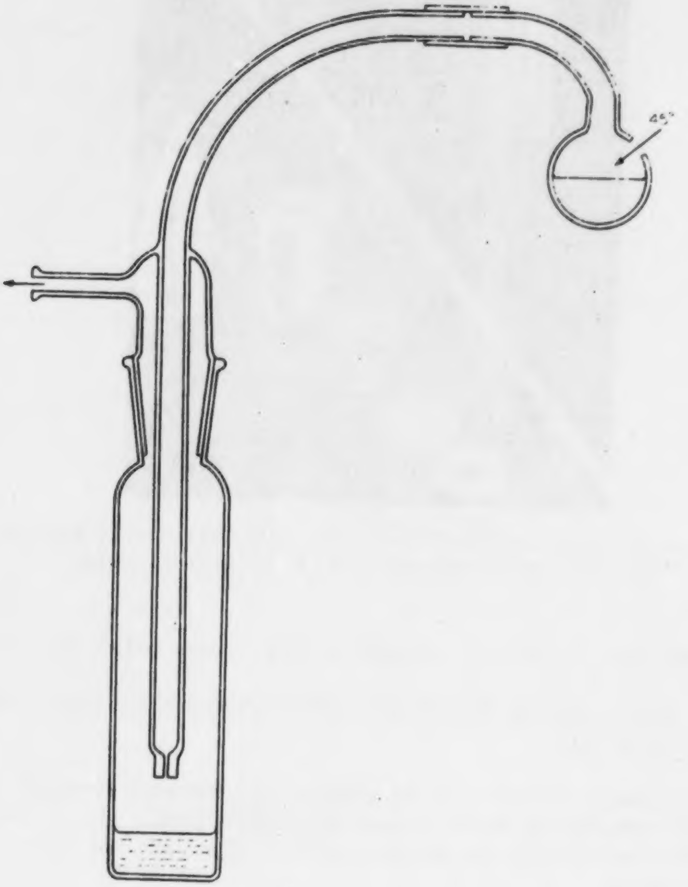

Figure 3.4.7 Porton impinger and pre-impinger (Davies, 1971)

is rated as 80-90% efficient for capture of single cells of Bacillus subtilis or Escherichia coli at an airflow rate of 55 liters per minute (Davies, 1971). A Multi-Slit Large-Volume Air Sampler (Figure 3.4.9) has been developed recently in which air is drawn at a rate of 500 liters per minute through eight radial slits. Particulates are impinged onto a film of culture medium which flows continuously over a rotating disc into an effluent container. The concentrating factor is reported to be as great as 100,000. The instrument has a high rate of efficiency and samples very large volumes of air in a short time.

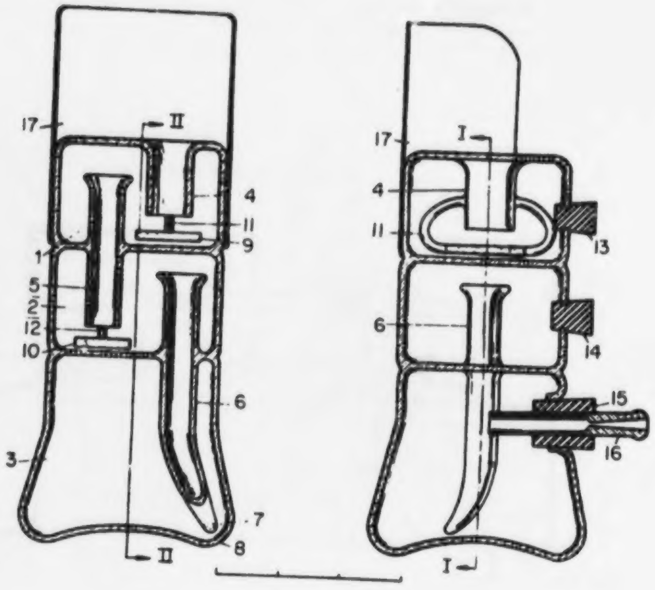

Figure 3.4.8 Sectional elevations, multi-stage liquid impinger, at right angles to each other in the directions I-II and II-II (Davies, 1971)

(1, 2, 3 - chambers or stages; 4 - air inlet tube; 5 - connection for airflow from stage 1 to stage 2; 6 - connection for airflow from stage 2 to stage 3; 7 - nozzle; 8 - annular well; 9, 10 - sintered glass discs held by curved glass rods 11 and 12; 13, 14, 15 - rubber bungs in access holes to chambers; 16 - connector for suction containing critical orifice; 17 - hemicylindrical metal shield)

Standard soil sampling methods for microbiological monitoring are not highly developed. Representative samples from agricultural soils may be collected by the method described by Bicking (1976) in which corings are taken to a depth of at least 10 centimeters from the center of random one-square-meter plots. The sampling device recommended is a 10 cm "bogey" hole cutter used on golf courses. The exact diameter of the core must be recorded so that the total surface area sampled can be calculated. It is recommended that ten to 20 cores representing at least 200 square centimeters be collected from each sampling area. If the soil is covered with grass or a cover crop, the sward should be cut and removed prior to sampling. Samples that cannot be analyzed immediately must be refrigerated in closed containers to prevent drying. Topographical features should be taken into consideration in

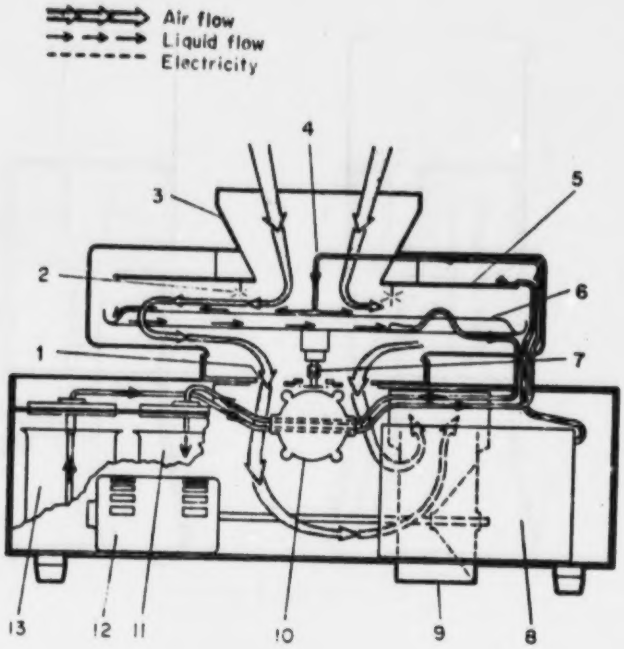

Figure 3.4.9 Diagramatic section, Litton LVS/10K air sampler (Davies, 1971)

(1 - airflow ports; 2 - corona needles; 3 - inlet duct; 4 - liquid input tube; 5 - high voltage plate; 6 - collection plate; 7 - multi-jaw coupling; 8 - high voltage power supply; 9 - blower; 10 - pumps; 11 - return reservoir; 12 - blower motor; 13 - fluid reservoir)

selecting sampling sites in areas subjected to spray irrigation of chlorinated sewage effluent or treated with sludge as well as in areas in the vicinity of sewage disposal plants, solid waste disposal facilities, rendering plants, and landfill areas.

Raw agricultural commodities grown in areas where the possibility of contamination with human enteric pathogens exists should be monitored by appropriate microbiologic procedures. Surface samples from crops such as potatoes, carrots, radishes, tomatoes, egg plant, etc., should be collected; internal as well as external samples are necessary in the case of lettuce, broccoli, cauliflower, celery, etc. The sample should be representative of the entire crop area. After compositing, representative aliquots should be preserved for the various tests to be performed.

3.4.1.2 Procurement and Acceptance Specifications--

Each item of equipment purchased should be tested in the laboratory under working conditions as soon as received to make certain that it meets the manufacturer's claims and the laboratory's specifications based on the work to be performed. Incubators, water baths, autoclaves, hot air ovens, deionization equipment, safety cabinets, etc., should be thoroughly checked for consistent performance before routine use (Ellis, 1976).

All special reagents such as stains, media supplements, diagnostic biologicals, buffers and other chemicals, etc., should meet or exceed CDC's current standards (CDC, 1969). The necessity for continuing reagent testing by the user laboratory was brought to light by a recent survey in which 14% to 27% of microbiologic reagents examined over a 2-year period gave unsatisfactory results. All new lots of reagents should be tested in parallel with reference quality control preparations, if available (CDC), as well as with a satisfactory lot in current use, with both positive and negative cultures. The proposed 4th edition of CDC's Recommended Specifications for Microbiological Reagents covers over 2000 different products (Suggs, 1973). Each reagent lot should be dated and stored at all times in accordance with the manufacturer's recommendations. Care should be exercised to avoid contamination. Performance testing should be repeated each time a new batch of reagents is prepared. Many laboratories go much beyond this. Gram staining solutions, for example, are tested at the beginning of each day with at least one gram-positive and one gram-negative organism as a control on performance. The Quality Control Supervisor should make certain that unsatisfactory substitutions are not made for specific brand products stipulated in test procedures.

Most laboratories today use commercially-prepared dehydrated or "ready to use" culture media for microbiologic work (Power, 1973). In spite of the fact that most lots are subjected to quality control tests by the manufacturer before release, contamination and unsatisfactory performance are still reported (Russell et al., 1969; Halstead et al., 1971). In one survey, of media collected from eight different laboratories, 46% of the chocolate agar plates failed to support the growth of Hemophilus influenzae or Hemophilus parainfluenzae (Barry and Feeny, 1967). Another laboratory discarded 5% of all media lots purchased over a period of time because of contamination, poor performance, etc. In another survey, however, 900 lots of 46 different media from two commercial suppliers tested over an 8-month period by a university medical school and a general hospital, revealed only four lots as unsatisfactory on the basis of performance, contamination, or physical properties (Power, 1973). Nevertheless, each new lot of media should be sterility-tested and tested for performance upon receipt and each time a new batch is prepared (Blazevic et al., 1976).

Quality control supervisors should make certain that performance failures with dehydrated commercial media are not due to error in final preparation or to improper storage. Errors in weighing, amount of water, pH measurement, and in addition of supplements, incomplete mixing, overheating during sterilization, and use of unclean glassware (residue of chemicals or detergent) must be avoided. Water should meet U.S. Pharmacopeia XIX requirements (U.S. Pharmacopeia, 1976). Aluminum foil for capping glassware for sterilization should be oil-free. The use of soft glass Pasteur-type disposable pipettes which release alkali and organic contaminants may also be responsible for errors in certain types of performance tests (Ellis, 1976). Loss of moisture from media during storage can cause performance failures. For example, it was found that unwrapped, poured petri dishes lost 7% moisture per week at 4°C, cellophane-wrapped dishes lost 2% per week, while those wrapped in polystyrene and stored in polystyrene containers lost only 0.5% per week under the same conditions (Power, 1973).

Performance tests on culture media and reagents must be conducted with standard control cultures with documented satisfactory performance by a reliable source. Such cultures are available from the American Type Culture Collection, Rockville, Maryland. Laboratories with the proper equipment and trained personnel may prepare a large number of lyophilized stocks from the official control cultures which, if properly stored, will retain viability with unchanged characteristics over a long period of time (Morton, 1973). A list of control cultures for use in performance tests on a variety of standard bacteriologic media and reagents together with a description of the correct reaction in each case has been compiled by Bartlett (1973) and is presented, with permission, in Appendix C. It is recommended that each batch of culture media, yeast extract, and peptone be checked by gas-liquid chromatography for the presence of fatty acids and that those containing excessive amounts be discarded. Standard reference toxins, antitoxins, antigens, antisera, and other diagnostic biologicals may be purchased from one of the reliable manufacturers listed in Section IX of the CDC Quality Control Manual for Microbiological Laboratories (Ellis, 1976). Procedures and reagents for quality control work in the highly specialized area of fluorescent antibody techniques are given in CDC manuals by Cherry et al. (1960) and Hebert et al. (1972).

The essentials of performance tests with equipment, culture media, reagents, and other supplies in the areas of mycology, virology, and parasitology are, in general, the same as those outlined above for bacteriology. Specific procedures, cultures, and materials required are outlined in two American Public Health Association publications - Diagnostic Procedures for Bacterial, Mycotic, and Parasitic Infections (Bodily et al., 1970) and Diagnostic Procedures for Viral and Rickettsial Infections (Lennette and Schmidt, 1969). Viral and fungal cultures are available from The American Type Culture Collection; parasitologic specimens may be obtained through the suppliers listed in the CDC Quality Control Manual for Microbiologic Laboratories (Ellis, 1976).

A logbook should be maintained for all quality control tests on all culture media, reagents, and other supplies. Name of product, lot number, manufacturer, date of receipt, and storage data should be logged in for all products upon receipt. Data on each performance test should include date, product, lot number, medium or reagent(s) used, type of test, standard control culture data, completion date, results (whether satisfactory or unsatisfactory) together with disposition of the lot or batch. All personnel, including those responsible for preparation of media, reagents, test cultures should sign the logbook. The Quality Control Supervisor should make certain that all performance tests are conducted according to the program schedule and protocols.

3.4.1.3 Instrument Checks and Calibration--

A wide variety of precision instruments and other types of mechanical equipment are necessary for diagnostic microbiology. New instruments must be properly calibrated and tested before use; instruments in use must be recalibrated at stated intervals. Instrument calibration and a regular check on all mechanical equipment is an important part of the quality control program. Serious errors can result from variation in incubator temperature, anaerobic jar failures, fluctuations in CO_2 levels in capneic incubators, etc. Cold rooms, walk-in incubators, and freezers should be equipped with Hi-Lo thermometers, recording thermometers, and alarm systems. A dry-ice box should be available in the event of a breakdown of mechanical freezers. Daily checks are recommended for incubators, water baths, hot blocks, refrigerators, freezers, anaerobic and carbon dioxide incubators. Autoclaves and centrifuges should be tested weekly. Safety cabinets should be checked for face velocity each month and filters should be replaced every six months (Russell, 1974).

Airborne contamination can be one of the main sources of error in diagnostic microbiology. Most laboratories today solve this problem through the use of laminar flow hoods or cabinets in combination with High Efficiency Particulate Air (HEPA) filters. HEPA filters regularly retain 99.9% or more of particles as small as 0.3 μm in diameter and thus remove most bacteria and some viruses from the airstream. The general principle of the laminar flow cabinet is a rapid "piston-like displacement" of all air in the cabinet by egress of filtered air from a whole wall or ceiling and withdrawal from the opposite side. Any particles entering from the outside are quickly swept away by the air flow before contamination can occur. In addition to the general type of cabinet, other hoods are available for special purposes. The Class I partial containment cabinet is designed to give maximum protection to the operator by drawing all air through front of cabinet across the work area and exiting it through filters at top of cabinet. The Class II partial containment cabinet protects both operator and experimental materials. The inward flow of air at front of cabinet protects the operator and the recirculation of air through the filters provides clean air over the work area. The Class III or absolute containment cabinet is a sealed unit which completely shields the work material from the external environment and the operator from any infectious agents associated with the experimental material. All manipulations are performed by the operator through sealed-in gloves which extend into the work area (Coriell, 1973a). A fourth cabinet, the horizontal flow cabinet, directs airflow from back to front of cabinet across the work area; it can be used only with noninfectious material and is advantageous for preparing petri dish media, tubing media, reagents, etc.

3.4.1.4 Experimental Design--

Standard methods are available for all diagnostic work the microbiology laboratory will be called upon to perform. Moreover, these procedures must be followed precisely and completely to obtain valid results. However, two aspects of sound experimental design should be emphasized - replicate samples and controls.

The need for replicate samples in experimentation has been well established and needs no further documentation here. In the more exact sciences, such as analytical chemistry, the use of duplicate samples is routine. In microbiology, however, replicates within a sample are more common than replicate samples. For example, in the titration of viruses with cell cultures, it is common to use 5 tubes at half-log dilutions or 10 tubes at log dilutions over a range of $10^{0.5}$ to 10^{7}. It would appear, however, that at least two samples should be used in every experiment even with samples collected by the most reliable sampling procedure.

Blanks, vehicle controls, and other forms of negative controls are usually included in laboratory procedures today. Positive controls, however, are often omitted. Changes in sensitivity of the test system can lead to grossly erroneous results if not checked with appropriate positive control preparations. The continuing sensitivity of any test system should not be taken for granted. Historical controls, if available, should also be taken into consideration in the assessment of results (Prier et al., 1973; Vera, 1971; Russell, 1974).

3.4.1.5 Standard Methods--

Standard methods in the field of diagnostic microbiology have been tested and refined over a period of many years. They are available in the following standard reference works:

Water and Wastewater -	Standard Methods for the Examination of Water and Wastewater, 14th Edition, 1975 (Rand et al., 1975)
	Handbook for Evaluating Water Bacteriological Laboratories - U.S. Environmental Protection Agency (Geldreich, 1975)
Bacteria, Fungi, & Parasites -	Diagnostic Procedures for Bacterial, Mycotic, and Parasitic Infections, 1970, APHA (Bodily et al., 1970)
	Manual of Clinical Microbiology, 2nd edition, 1974, ASM (Lennette et al., 1974)
	Diagnostic Microbiology (Bailey and Scott, 1970)
	Bergey's Manual of Determinative Bacteriology, 8th Ed. (Buchanan and Gibbons, 1974)

Viruses & Rickettsiae	- Diagnostic Procedures for Viral and Rickettsial Infections, 4th Edition, 1969 (Lennette and Schmidt, 1969)

Basic techniques may be found in:

Methods in Microbiology, Vols. 1-9 (Davies, 1971).

Methods in Virology (Maramorosch and Koprowski, 1967)

Sample protocols for bacterial and viral assays are given in the following pages.

A Procedure Manual which contains complete protocols for each diagnostic test performed by the laboratory as well as procedures for all ancillary work such as media preparation, reagent testing, etc., is essential. A "loose-leaf" type which can be revised and updated easily is ideal. It is a major responsibility of the Quality Control Supervisor to make certain that this manual is complete, up-to-date, and is followed without variation at all times (Bartlett, 1973; Russell, 1974).

3.4.1.6 Proficiency Testing--

Proficiency testing in the area of diagnostic microbiology involves the use of standards or unknowns for identification by the laboratory staff (LaMotte, 1973; Prier, 1973; Wilson, 1973). The ability of a laboratory to correctly identify samples of this type in a consistent manner over a period of time is probably the best assurance that the quality control program, for that particular area at least, is achieving its objectives.

Two general types of standards are used in proficiency testing - external standards and internal standards. External standards are unknowns made up and distributed by the quality control section of an organization such as the College of American Pathologists, American Society of Clinical Pathologists, or the Center for Disease Control. Internal standards are unknowns prepared by the laboratory for evaluating the performance of their own personnel. Both types of standards have their own special advantages and drawbacks. External standards are prepared by specialists in the area of quality control. However, laboratories tend to work more carefully and to expend extra effort when such unknowns are announced. Internal unknowns are prepared on a much smaller scale and usually have less documentation than external unknowns. Internal unknowns, however, can be slipped unannounced into the regular flow of specimens and thus are tested routinely or without special attention. Moreover, they can be tailored to appraise special efforts to improve performance of weak areas of the laboratory. The preparation and preservation of satisfactory unknowns in certain areas require great care and considerable "know how". For example, considerable difficulty has been encountered in maintaining viability of small numbers of E. coli in simulated drinking water samples for distribution to laboratories. The problem was finally solved with the discovery that a formate lactose glutamate medium (without lactose) with boric acid added to a final concentration of 1.8% would preserve viability for 7 to 10 days. The suspension is diluted 3:200 for examination (Gray and Lowe, 1976). Many laboratories use both external and internal standards in their proficiency testing program thus profiting by the advantages of each.

The number of unknowns employed and the frequency of testing varies from laboratory to laboratory. Some laboratories use as few as two internal unknowns per month, others include several each week. Internal unknowns are rotated so that all areas of the laboratory are checked periodically. Proficiency testing of clinical laboratories subject to interstate commerce regulations is under the jurisdiction of the Center for Disease Control. Private laboratories, however, may set their own schedule for external standards testing. One large teaching hospital laboratory receives four sets of unknowns a year from each of two large private accreditation organizations - Survey Program of the College of American Pathologists and the Check Sample Program of the Commission on Continuing Education of the American Society of Clinical Pathologists. Government agency laboratories are eligible also for participation in the CDC proficiency testing program.

Proficiency testing results with both internal and external unknowns are discussed with laboratory personnel by the supervisor. Where areas of deficiency have been revealed, remedial measures are instituted. Special on-the-job training, laboratory courses and workshops are some of the methods that may be used to increase the quality of performance (Russell, 1974).

EPA is now in the process of certifying laboratories performing analyses of drinking water (Geldreich, 1975).

EXAMPLE: BACTERIAL ASSAY

Multiple-Tube Fermentation Technique - Coliform Group: Standard Total Coliform Most Probable Number (MPN) Tests

Purpose of Study

- Ascertainment of the presence or absence of coliform organisms in water and estimation of their density in terms of the Most Probable Number as an aid in establishing the sanitary quality of the water.

- The coliform group comprises all of the aerobic and facultative anaerobic, Gram-negative, non-sporeforming, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35°C.

Design of Experiment

- Tests:

Presumptive Test
    Positive test - An indication of coliform organisms in sample
    Negative test - Absence of coliforms

Confirmed Test
Positive test - Additional evidence for presence of coliforms
Negative test - Absence of coliforms

Completed Test
Positive test - Proof of presence of coliform organisms in sample
Negative test - Absence of coliforms

- Diagnostic Media:

Presumptive Test
Lactose broth or lauryl tryptose broth

Confirmed Test
Lactose broth or lauryl tryptose broth
Brilliant green lactose bile broth

Completed Test
Lactose broth or lauryl tryptose broth
Brilliant green lactose bile broth
Endo medium or Eosin methylene blue (EMB) medium
Agar slant

- Inoculum and Number of Fermentation Tubes

U.S. Environmental Protection Agency Standards for water:
five tubes of Presumptive medium, 10 ml or 100 ml of water sample each

Other waters presumed to be of drinking-water quality:
five tubes, at least, in each of at least three dilutions

Conduct of Experiment

- Presumptive Test: Inoculate a series of fermentation tubes ("primary" fermentation tubes) with appropriate graduated quantities (multiples and submultiples of 1 ml) of the water to be tested. Bottles to contain 100-ml sample portions should be prewarmed in a water bath at 35°C; after adding the sample, mix thoroughly and aseptically add a sterile fermentation vial. The concentration of nutritive ingredients in the mixture of medium and added portion of the sample must conform to the requirements given in Section 905C, Media Specification, Media 2 and 3, in reference at end of this protocol. The portions of water sample used for inoculating the lactose or lauryl tryptose broth fermentation tubes will vary in size and number with the character of the water under examination, but in general should be decimal multiples and submultiples of 1 ml. These should be selected as outlined under Design of Experiment for types of water indicated. Incubate the inoculated fermentation tubes at 35 ± 0.5°C.

QUALITY CONTROL -- The accuracy of any single test is dependent upon the number of tubes used.

- Confirmed Test: Lactose broth or lauryl tryptose broth may be used for the primary fermentation; however, lauryl tryptose broth is recommended when experience shows a high proportion of false positive tubes of lactose broth.

Use brilliant green lactose bile broth fermentation tubes for the Confirmed Test.

Procedure: Submit all primary fermentation tubes showing any amount of gas at end of 24 hours of incubation to the confirmed test. If active fermentation appears in the primary fermentation tube before expiration of the 24-hour period of incubation, it is preferable to transfer to the confirmatory medium without waiting for the full 24-hour period to elapse. If additional primary fermentation tubes show gas production at the end of 48-hour incubation, these too shall be submitted to the confirmed test.

Alternative procedure: Where three or more multiple portions of a series of three or more decimal dilutions of a given sample are plated submit to the Confirmed Test all tubes of the two highest dilutions (smallest volumes) of the original samples showing gas formation in 24 hours.

All tubes producing gas in 24 hours that have not been submitted to the Confirmed Test must be recorded as containing organisms of the coliform group -- that is, as positive - even though all the confirmed tests actually performed yield negative results.

Submit to the Confirmed Test all tubes of all dilutions of the original sample in which gas is produced only at the end of 48 hours.

If less than three portions of any dilution (volume), or if a series of less than three decimal dilutions of the original sample is plated submit all tubes producing gas at 24 and 48 hours to the confirmed test.

Procedure with brilliant green lactose bile broth: Either 1) gently shake or rotate primary fermentation tube showing gas and with a sterile metal loop, 3 mm in diameter, transfer one loopful of medium to a fermentation tube containing brilliant green lactose bile broth, or 2) gently shake or rotate primary fermentation tube showing gas and insert a sterile wood applicator at least 2.5 cm (1 inch) into the medium. Promptly remove and plunge applicator to bottom of fermentation tube containing brilliant green lactose bile broth. Remove and discard applicator.

Incubate the inoculated brilliant green lactose bile broth tube for 48 ± 3 hours at 35 ± 0.5°C.

- Completed Test: The Completed Test is used as the next step following the Confirmed Test. It is applied to the brilliant green lactose bile broth fermentation tubes showing gas in the Confirmed Test. The procedure is as follows.

Streak one or more Endo or eosin methylene blue plates from each tube of brilliant green lactose bile broth showing gas, as soon as possible after the appearance of gas.

Incubate the plate (inverted, if with glass or plastic cover) at 35 ± 0.5°C for 24 ± 2 hours.

The colonies developing on Endo or eosin methylene blue agar may be described as typical (nucleated, with or without metallic sheen); atypical (opaque, unnucleated, mucoid, pink after 24-hour incubation), or negative (all others). From each of these plates fish one or more typical well-isolated coliform colonies or, if no typical colonies are present, fish two or more colonies considered most likely to consist of organisms of the coliform group, transferring each fishing to a lactose broth or a lauryl tryptose broth fermentation tube and to a nutrient agar slant.

The use of a colony counter is recommended to provide optimum magnification when colonies are fished from the plates of selective medium.

QUALITY CONTROL -- It is essential that the Endo or EMB plates be so streaked as to insure the presence of some discrete colonies, separated by at least 0.5 cm from one another. Careful attention to the following details when streaking plates will result in a high proportion of successful isolations if coliforms are present: Use an inoculating needle slightly curved at the tip; tap and incline the fermentation tube to avoid picking up membrane or scum on the needle; insert end of needle into liquid in tube to a depth of approximately 5.0 mm; streak plate by contacting agar surface with curved section of needle only so that agar will not be scratched or torn. When transferring colonies, choose well-isolated colonies separated by at least 0.5 cm and barely touch surface of colony with a flame-sterilized, air-cooled transfer needle, to minimize danger of transferring a mixed culture.

Observations and Tests

- Presumptive Test: At end of 24 ± 2 hours, shake each tube gently and examine it and, if no gas has formed and been trapped in the inverted vial, repeat this step at the end of 48 ± 3 hours. Record the presence or absence of gas formation at each examination of the tubes, regardless of the amount.

Interpretation: Formation within 48 ± 3 hours of gas in any amount in the inner fermentation tubes or vials constitutes a positive Presumptive Test.

The absence of gas formation at the end of 48 ± 3 hours of incubation constitutes a negative test. An arbitrary limit of 48 hours for observation doubtless excludes from consideration occasional members of the coliform group that form gas very slowly and are generally of limited sanitary significance; for the purpose of a standard test based on the definition of the coliform group, exclusion of these occasional slow gas-forming organisms does not compromise the value of the test.

QUALITY CONTROL -- Appearance of an air bubble in inner fermentation tubes or vials must not be confused with actual gas production. If the gas is formed as a result of fermentation, the broth medium will become cloudy. Active fermentation may be shown by the continued appearance of small bubbles of gas throughout the medium outside the inner vial when fermentation tube is gently shaken.

- Confirmed Test: The formation of gas in any amount in the inverted vial of the brilliant green lactose bile fermentation tube at any time within 48 ± 3 hours constitutes a positive Confirmed Test.

- Completed Test: The agar slants and secondary broth tubes are incubated at 35 ± 0.5°C for 24 ± 2 or 48 ± 3 hours if gas is not produced in 24 hours. Gram-stained preparations from those agar slant cultures corresponding to the secondary lactose broth tubes that show gas are examined microscopically.

Interpretation: The formation of gas in the secondary lactose broth tube and the demonstration of Gram-negative, non-sporeforming, rod-shaped bacteria in the agar culture may be considered a satisfactory Completed Test, demonstrating the presence of a member of the coliform group in the volume of sample examined.

If, after 48 ± 3 hours, gas is produced in the lactose and no spores or Gram-positive rods are found on the slant, the test may be considered completed and the presence of coliform organisms demonstrated.

QUALITY CONTROL -- A Gram-positive and a Gram-negative culture should be used as controls for the Gram-staining process.

Data Handling and Validation

- Record permanently analytical data in meaningful, exact terms.

- Report data in proper form to an information storage facility for future use.

- All laboratory personnel must agree upon precise rules for the use of significant figures, rounding off numbers, and arithmetic operations.

- Use bound laboratory record books and preprinted report forms. Laboratory records should be readily available for inspection and held on file for at least two years. The multi-copy report forms are highly recommended for recording all information from sample collection to calculation of results. One copy of these forms is then forwarded to the appropriate office for computer data entry.

- A study on analytical quality control methods for use in validating microbiological data by a group of researchers at the Robert S. Kerr Water Research Center at Ada, Oklahoma, demonstrated that precision control charts are a useful tool for precision but they cannot measure accuracy; that is, the data can be precise and still be inaccurate. At least duplicate tests must be performed for each sample. The data must be plotted on an everyday basis and problems must be rectified immediately. Data from the same waters under study should be used to construct the control charts.

References

- Bordner, R. H. 1973. Quality control: A state-of-the-art. Proceedings of the First Microbiology Seminar on Standardization of Methods. Office of Research and Monitoring, U.S. Environmental Protection Agency, EPA-R4-73-022, Washington, D.C., pp. 170-194.

- Rand, M. C., A. E. Greenberg, and M. J. Taras (eds.). 1975. Standard Methods for the Examination of Water and Wastewater, 14th Edition. American Public Health Association-American Water Works Association-Water Pollution Control Federation, Washington, D.C., pp. 968-975.

EXAMPLE: WATERBORNE ENTERIC VIRUS ASSAY

Purpose of Study

- Detection of waterborne enteric viral pathogens

Design of Experiment

- Entire sample-concentrate must be inoculated into indicator hosts (see Section 3.4.1.1)

- Indicator hosts
  Cell cultures: Primary African Green monkey kidney*, and human embryonic kidney*
  Suckling mice: less than 24 hours old

- Number of mice or cell cultures:
  Suckling mice - at least two litters
  Cell cultures - sufficient for remainder of sample after mouse inoculation

Conduct of Experiment

- Suckling mice: Mice are inoculated with a portion of the original sample-concentrate by the intracerebral (0.02 ml) and intraperitoneal (0.05 ml) routes. Animals are then observed daily for 14 days.

  QUALITY CONTROL -- A vertical-flow laminar-air hood is used for all virus assay procedures.

  Temperature, humidity, and all other environmental conditions in animal rooms should be maintained at proper level for suckling mice and dams.

- Cell cultures: Remaining sample-concentrate is inoculated onto monolayer cultures of two cell types. Inoculum must not exceed 0.06 ml/cm^2 of cell surface area. After a 2-hour adsorption period at 36° ± 0.5°C, inoculum is decanted and maintenance medium is added to cells. Cultures are then incubated at 36° ± 0.5°C and observed microscopically, daily for 3 days and then periodically for 14 days. Medium is not changed during observation period unless required to maintain healthy cells.

  QUALITY CONTROL -- Negative (uninoculated) controls must be included (both cell culture types as well as mice).

  Cell cultures must be free from Mycoplasma contaminants and "passenger viruses."

  All tissue culture containers should be permanently labelled.

  Replicate assays should be run for each cell culture type.

  Glassware, pipettes, media, and reagents for cell cultures must be of tissue culture grade. Media and reagents should be performance-tested before use.

  Incubators must be monitored carefully to make certain temperature is maintained within prescribed limits.

  Microscopes, incubators and any other precision equipment employed should be checked frequently and adjusted or recalibrated if necessary.

Observations and Tests

- Suckling Mice: Mice showing no pathology by 14th day of first passage are regarded as negative for Coxsackie virus group A.

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* Other cultures may be used if evidence is available to show that "cells have equivalent spectral sensitivity for enteric virus replication."

Mice exhibiting any pathologic changes (e.g., flaccid or spastic paralysis of the extremities) are sacrificed and passaged a second time in suckling mice according to procedures and analyses outlined in Diagnostic Procedures for Viral and Rickettsial Diseases (Melnick, J. L., H. A. Wenner, and L. Rosen. 1964. The Enteroviruses. In: Diagnostic Procedures for Viral and Rickettsial Diseases, Lennette, E. H., and N. J. Schmidt (eds.), 3rd Edition, American Public Health Association, New York, N.Y., pp. 217-218).

- Cell Cultures: Cell cultures showing no cytopathic effects (CPE) by the 14th day are frozen and thawed once for re-passage. Harvest-fluids from a single cell culture type are pooled and 20% of the volume is inoculated onto a second cell culture monolayer of the same cell type. Cell cultures negative for CPE on 14th day of the second passage are considered negative for virus.

All cultures positive for CPE are confirmed for presence of virus by additional passages.

Virus isolates are identified by appropriate serologic procedures (Diagnostic Procedures for Viral and Rickettsial Diseases, Lennette, E. H., and N. J. Schmidt (eds.), 3rd Edition, American Public Health Association, New York, N.Y., 1964).

Reference

- Rand, M. C., A. E. Greenberg, and M. J. Taras (eds.). 1975. Standard Methods for the Examination of Water & Wastewater, 14th Edition. American Public Health Association-American Waterworks Association-Water Pollution Control Federation, Washington, D.C., pp. 968-975.

3.4.2 Microorganisms - Mutagenicity Testing

An important milestone in the history of mutation research was reached in 1947, when for the first time, a chemical, mustard gas, was shown to be capable of artificially inducing cell mutation in an experimental test organism, Drosophila melanogaster (Auerbach et al., 1947). Since this initial discovery, a whole host of chemicals have been found to be mutagenic in a variety of animals and other test organisms.

In view of the fact that mutation tests in mammals are time-consuming and relatively expensive, researchers early turned to simpler forms of life with a more rapid generation rate as a test system. Microorganisms (bacteria, yeasts, mold, protozoa), mammalian cell cultures, and insects (Drosophila melanogaster strains mainly) are widely used today (Anon., 1975). However, opinion is sharply divided regarding the value of mutagenicity testing with nonmammalian systems. One school contends that results of value in predicting the likelihood of mutagenicity in man can be obtained only with the intact animal where the chemical is subject to metabolic and detoxification mechanisms (WHO, 1971; Anon., 1972). Others take the stand that mutations result basically from alterations in DNA which is essentially the same for all organisms; moreover, metabolic and detoxification mechanisms can be brought to bear on the test compound in microbial or cell culture assays through the use of microsomal systems in vitro (Ames and Yanofsky, 1971; Ames et al., 1973a). Mutagenicity testing takes on added significance with the accumulation of evidence that most mutagens are also carcinogens and some authorities regard mutagenicity testing as practically equivalent to carcinogenicity testing (Ames and Yanofsky, 1971; McCann et al., 1975).

Genetic change can be brought about in many different ways and no single mutagenicity system has been developed to date which will detect all types of mutations. Major mechanisms involved are: Gene mutations and chromosomal mutations. Gene mutations may be point mutations (base-pair substitutions, frameshift mutations, small deletions or insertions) or small multilocus mutations. Chromosomal aberrations may be ploidy changes or chromosomal breaks and/or misreplication and/or misrecombination effects (U.S. EPA, 1977). Consequently, most authorities today recommend a tier system in mutagenicity testing, each tier consisting of a battery of tests in an effort to detect all of the kinds of mutations that can be induced by a chemical:

Tier 1 - Microbial cultures
Tier 2 - Mammalian cell cultures and/or Drosophila strains
Tier 3 - Mammals

Microbial systems most commonly recommended for Tier 1 are:

- Host-mediated assay with Salmonella typhimurium
- Ames test - Salmonella typhimurium in vitro with and without microsomal activation
- Mitotic gene conversion test with Saccharomyces cerevisiae
- Repair-deficient E. coli with activation (Anon., 1975; U.S. EPA, 1977; Huisingh, 1976; Flamm, 1974; Zimmermann, 1975)

The bacterial system can be used to detect gene mutations (base-pair substitution mutation, frameshift mutation, and stimulation of DNA repair). Saccharomyces cerevisiae can be used to detect mitotic recombination and mitotic gene conversion as well as forward and reverse gene mutations. Decisions are made at the end of each tier test regarding further testing. Organizations requiring definitive tests in mammals may use the tier system to establish testing priorities.

3.4.2.1 Methods--

- The Host-mediated Assay

Although microbial assays have a number of advantages over mammalian tests - short generation time, large cell population, short test period, great sensitivity, large range of detectable compounds, simplicity, economy, etc. - the usual in vitro tests do not detect, and may give misleading results, with:

- Chemicals (promutagens) which must undergo metabolic transformation in the host to become mutagenic (false negative results)

- Compounds which are detoxified by the host (false positive results)

The host-mediated assay developed by Legator and coworkers (Gabridge and Legator, 1969; Legator and Malling, 1971; Legator, 1976), is designed to correct these shortcomings and to bridge the gap between the in vitro assay and definitive mutagenicity tests in animals.

In the host-mediated assay, the test organism is injected into an animal undergoing treatment with a suspected mutagen. After a period of several hours, the indicator organisms are withdrawn from the peritoneum and plated on appropriate media to detect any induced mutants (Figure 3.4.10). Since the chemical is subjected to both metabolic and detoxification systems of the host, the possibility of false-negative and false-positive results is reduced to a considerable degree. An in vitro test in which the test chemical acts on the indicator organism directly is included as a control on the host-mediated effect.

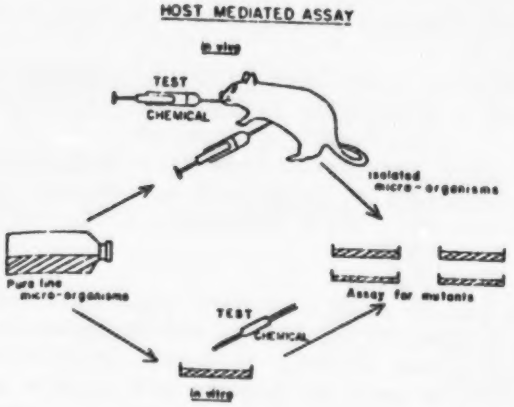


Figure 3.4.10 The technique of host-mediated assay involves the comparison of the mutagenic power of a chemical when tested in vitro and when tested in the peritoneum of the rodent after oral administration. It thus gives a measure of in vivo activation and detoxication of the compound (Clayson, 1973)

An example of a protocol for the host-mediated assay is given in the following pages.

EXAMPLE: HOST-MEDIATED ASSAY

Purpose of Assay

- Mutagenicity testing

Design of Experiment

- Test Culture:

  Salmonella typhimurium histidine auxotrophs (His$^-$) Strains TA-1537, TA-98, TA-100 (Ames)
  Escherichia coli Strains 1212/343/113
  Saccharomyces cerevisiae
  Neurospora crassa

- Host: Swiss albino mice (25 to 30 g) usual host

- Dosage of Test Substance

Recommended doses: Maximum tolerated dose (MTD) (highest dose); intermediate concentration (intermediate dose); maximum use concentration (lowest dose). If the usage level is not known, second and third doses can be one log and two logs, respectively, lower than the MTD.

- Vehicle: Corn oil, ethanol, and dimethylsulfoxide are suitable vehicles for substances not soluble in water

- Controls: Positive, vehicle, and untreated controls should be included in each assay. Positive control compound should be a known pro-mutagen of the same chemical class as the test compound

Conduct of Experiment (S. typhimurium strains)

- Test compound may be administered to the host by any route other than intraperitoneal.

QUALITY CONTROL -- Test chemicals must be collected by a statistically sound method to ensure that sample is representative of entire batch or lot.

- Tester (indicator) strains are injected intraperitoneally to separate animals about 4 hours after treatment with test compound in acute tests. The inoculum consists of 2 ml of a suspension containing 3×10^8 to 5×10^8 cells per ml at log phase of growth.

QUALITY CONTROL -- Test compound should be of highest purity unless technical grade, mixture, or formulation is to be tested.

Test animals must be healthy and should be selected randomly for various parts of test.

- Recovery of Indicator Organism from Host: Three hours after administration of indicator organisms, 1 to 2 ml of sterile saline is injected into the peritoneal cavity and animals are sacrificed for aseptic removal of the peritoneal exudate.

QUALITY CONTROL -- Strict aseptic technique must be used for parenteral administration of both test chemical and indicator organisms.

Tester strains must be obtained from a reliable source and monitored frequently to make certain that no changes in essential test genetic markers have occurred.

- Detection of Mutants:

1) Petri dishes with nutrient medium containing only 40 μg/ml histidine (minimal agar) are streaked with 0.2 ml of undiluted exudate for detection of host-mediated mutants. Five plates are streaked for each animal.

QUALITY CONTROL -- The streaking technique used must ensure that organisms will develop into discrete colonies which can be counted accurately.

All culture operations should be carried out in a biological cabinet equipped with HEPA filters to prevent contamination by ambient airborne microorganisms.

2) To determine total number of surviving bacteria, dilutions of exudate are streaked in same manner, in triplicate, on tryptone-yeast agar (complete agar).

3) A parallel in vitro test is carried out to ascertain if test chemical will induce mutation without intervention of a host (mutagen instead of pro-mutagen). A 0.1 ml sample of an overnight broth culture of the tester strain is added to 2 ml of cooled molten agar (0.6%) and poured over a minimal agar base plate, and test chemical spotted on center of dish. Positive and negative (vehicle and untreated) controls must be included.

4) All plates are incubated inverted at 37°C for 24 or 48 hours.

QUALITY CONTROL -- Incubators must be monitored during tests to make certain that the desired temperature is maintained uniformly throughout the appliance.

Observations and Tests

- Mutant histidine revertants (His^+) only will grow on the minimal agar plates (trace of histidine) streaked with exudate. The colonies can be counted after 48 hours at 37°C.

QUALITY CONTROL -- Any contaminated plates must be excluded from the assay and the samples recultured.

- All surviving organisms (His^- originals and His^+ mutants) will grow on the complete medium, and colonies are counted after 24 hours at 37°C.

Mutation frequency (MF) is calculated for each sample:

$$MF = \frac{His^+ \text{ mutants/ml}}{\text{CFU/ml in undiluted exudate}}$$

(CFU = Number of colonies)

$$MF_t/MF_c = \frac{\text{MF of treated sample}}{\text{MF of control sample}}$$

- If mutants (His$^+$) are induced in the in vitro test, colonies will appear in a circle around the test sample.

- Comparison between direct effect of test substance on indicator organisms (in vitro test) and effect of animal (host-mediated effect) indicates whether or not the mammal can detoxify the compound or metabolize it with the formation of one or more mutagens.

- A negative result is not conclusive proof that a substance is not a mutagen or promutagen since not all mutagenic mechanisms are detectable by the tester strains now available. Also, although less likely, the host employed may not be able to metabolize a given promutagen with the formation of one or more mutagens. In spite of this weakness the host-mediated assay with *S. typhimurium* auxotrophs has been widely used with excellent results and is recommended by most experts for mutagenicity testing.

Data Collection and Analysis

- All results will be recorded on specially designed data sheets. QUALITY CONTROL -- All data sheets will be dated and signed by operator(s) conducting test. Data will be subjected to appropriate statistical analysis.

- Assays employing other acceptable indicator organisms are conducted in essentially the same manner as described above with appropriate minimal and complete nutrient media and inocula.

- Ray et al. (1973) found that the host-mediated assay using *Salmonella typhimurium* tester strains was considerably more sensitive in detecting mutagenesis with ethylmethanesulfonate (EMS) in random-bred Swiss CD-1 mice than the dominant lethal or cytogenetic assays. Relative sensitivities of three assays as determined by dose-response curves and no-effect dose levels were as follows:

Dominant lethal effects not evident until	150 mg/kg used
Cytogenetic test - no significant breaking of somatic cell chromosomes in bone marrow cells until	120 mg/kg used
Host-mediated assay - statistically-significant response detected at	35 mg/kg level

The second major advance in microbial mutagenicity testing methodology was the development by Ames and coworkers of a microsomal activation system which is capable of detoxifying or metabolizing potential mutagens in vitro. The preparation, obtained from the livers of rats induced with Aroclor 1254, can be mixed directly with cofactors, test chemical, bacteria, and culture medium in a single petri dish test system (Ames et al., 1973b; Ames et al., 1975). In addition to being simple, rapid, and inexpensive, the system is also extremely sensitive. Strong mutagens can be detected at levels as low as a few nanograms.

A sample protocol for the Ames Test is on the following pages.

The complete battery of Ames strains "are able to detect almost all mutagens", according to the authors. The only additional test required is an excision repair test. It should be borne in mind, however, that in the Ames test the test chemical is subjected to a certain fraction of the liver induced with a single chemical whereas in the host-mediated assay the complete metabolic and detoxification systems of the intact mammal are brought to bear upon the chemical. Nevertheless, the Ames test is highly regarded and is an almost unanimous choice of the experts for Tier 1 testing.

Other mutagenicity tests in recent use are outlined in Table 3.4.1.

3.4.2.2 Media, Reagents and other Supplies--

All media, reagents, and other materials should be tested for performance upon receipt and when each batch is prepared. Microsomal activation preparations together with cofactors must be tested with known promutagens before use and at intervals during storage. Performance specifications are given in the various test methodologies referred to above.

3.4.2.3 Instrument Calibration and Checks--

Incubator temperature should be checked at the beginning and end of each day. Centrifuges, freezers, refrigerators, autoclaves and all other equipment should be checked regularly according to the schedule given in Appendix C.

3.4.2.4 Experimental Design--

All mutagenicity assay systems employed should have a satisfactory performance documentation in the scientific literature. The test system must also be reproducible; identical results should be obtained by various laboratories with the same compounds. The system should not give false negative tests and few false positive results (de Serres, 1974). Each test system should be calibrated before routine use with known mutagens and negative chemicals (U.S. EPA, 1977). The spot test technique as well as the standard plate method should be used in each assay.

Test chemical specimens should include technical grades and formulations as well as purified preparations.

Dosage ranges vary somewhat depending upon the type of assay system and on the individual investigator. In the Ames test, the maximum dose is usually set at 500 μg (or the highest non-inhibitory level); doses as low as a few nanograms of strong mutagens are sufficient to induce mutations. Ames systems and types of mutagenic activity detected are shown in Table 3.4.2 (Legator, 1976).

In the host-mediated assay, at least three doses are used initially in screening chemicals. Dose levels suggested for several types of chemicals are given in Table 3.4.3.

TABLE 3.4.1. OTHER MUTAGENICITY TESTS

Assay	Strain Used	Experimenter
Mitotic gene conversion	*Saccharomyces cerevisiae* Strain D-4	Zimmermann (1973, 1975) Brusick and Andrews (1974) Ong et al. (1977)
DNA repair	*Escherichia coli* P3478 polA$^-$	Slater et al. (1971)
	E. coli W3110 pol$^+$	
Dominant or recessive lethal test	*Neurospora crassa*	Webber and de Serres (1965) de Serres and Malling (1971)
Host-mediated forward mutation	*N. crassa*	Legator and Mailing (1971)
Forward mutation (Canavanine resistance)	*S. cerevisiae*	Zeiger and Brusick (1971)
Host-mediated assay	*S. cerevisiae*	Fahrig (1975)
Chlorate reduction	*Escherichia coli* *Salmonella typhimurium*	Ames and Yanofsky (1971)
Forward mutation	*S.* typhimurium	Ames and Yanofsky (1971)

TABLE 3.4.2 GENE MUTATION DETECTION SYSTEMS (Legator, 1976)

Gene Alterations	*S. typhimurium* (histidine auxotroph)	*N. crassa* (adenine 3 locus)	*E. coli* 343/113	*S. cerevisiae*	Chinese Hamster	Murine Leukemia
Types detected:						
Mutations forward	No	Yes	Yes	Yes	Yes	Yes
Reverse	Yes	Yes	Yes	Yes	Yes	Yes
Mitotic gene conversion and recombination	No	No	No	Yes	No	No
Ease of detecting genetic events	Excellent	Fair	Excellent	Fair	Good	Good
Genetic validity of detected change	Established	Established	Established	Established	Questionable	Questionable
Growth division in host as compared to in vitro	Similar	No growth or division	Similar	Slight growth; no division	No growth or division	Similar
Spontaneous mutation frequency in host as compared to in vitro	Similar					Similar
Ability to localize genetic effect in host	With difficulty	With difficulty	Fair	Fair	With difficulty	With difficulty
Utility	Good	Questionable	Good	Good	Questionable	Good

TABLE 3.4.3 DOSE LEVELS FOR HOST-MEDIATED ASSAY

Chemical	Highest	Intermediate	Lowest
Environmental	LD50	LD50/5-LD50/10	LD50/50-LD50/100
Drugs	Toxic but permits survival of most animals	Intermediate between high and low doses	Close to pharmacologic threshold of drug
Drugs	LD5	Intermediate between high and low doses	Use-dose of drug
Drugs	5.0 g/kg*	500 mg/kg*	50 mg/kg*
General	MTD*	Intermediate between high and low doses	Maximum use level
General	MTD*	MTD/10*	MTD/100*

* If maximum use level or dose is not known, ten animals per dose level per indicator organism are usually employed in the host-mediated assay (Green et al., 1976). Dose response curves should be obtained in all cases where mutagenicity has been detected in the screening test. Toxicity data should be included in the mutagenicity test report on each compound tested.

EXAMPLE: MICROBIAL ASSAY (AMES TEST)

Purpose of Study

- Mutagenicity determination

Design of Experiment

- Test culture:

Salmonella typhimurium
Strains TA-1535, TA-1537, TA-98 and TA-100

Escherichia coli
Strains WP_2 $uvrA^-$, W3110/$polA^+$, P3478/$polA^-$

Yeast
Strain D4

PROPOSED TEST SCHEME FOR A SINGLE CHEMICAL

Nonactivation	Activation (Rat Liver)
Positive Controls (2)	Positive Controls (2)
Solvent Controls (3)	Solvent Controls (3)
Test Level 1 (1)	Test Level 1 (1)
2 (1)	2 (1)
3 (1)	3 (1)
4 (1)	4 (1)
5 (1)	5 (1)

- Replications: Each solvent control set will be performed in duplicate; each positive control and each test material will be performed with one plate for each of the concentrations. The assays will be performed under both nonactivation and activation conditions as outlined above. The total sum of plates required for the evaluation is also given.

- Chemical Levels: The proposed chemical range will consist of the following five concentrations:

Dose range	µl/plate (liquid)	µg/plate (solid)
1	0.001	1
2	0.05	10
3	0.5	100
4	1	250
5	5	500

Adjustments to either higher or lower concentrations will be made to accommodate variations in toxicity.

Conduct of Experiment

- Strain Culture: The strains employed will be grown overnight at 37°C on a shaker in complete medium.

QUALITY CONTROL -- Before the use of any test culture, samples of each culture will be removed and monitored for the appropriate marker per Table 1. Genetics of tester strains are given in Table 2.

- Activation System: Assays conducted in the presence of a microsome activation system will be performed as outlined above. The activation system will consist of the components listed in Table 3 and will use 9,000 x *g* supernatant from hepatic homogenates from the same species and strain of animals employed in associated carcinogenesis experiments (either mice or rats). The animals used to provide the hepatic tissue will be pretreated with Aroclor 1254 (Ames et al., *Mutation Res.* *31*:347, 1975) to induce the microsomal enzyme activity.

QUALITY CONTROL -- All samples of 9,000 x *g* supernatant will be monitored for protein content and P-450/P-448 activity. These latter measurements are

TABLE 1. MICROBIAL STOCK MONITORING PROCEDURES

Strain	Genus	Markers Monitored	Resistance to: Ampicillin[a]	Resistance to: Crystal Violet[b]	Spot Test Reversion[c]
TA-1535	*Salmonella*	his G, rfa	-	-	MNNG
TA-1537	*Salmonella*	his C, rfa	-	-	QM
TA-100	*Salmonella*	his G, rfa, Amp^R	+	-	MNNG
TA-98	*Salmonella*	his D, rfa, Amp^R	+	-	NF
WP$_2$ uvrA$^-$	*Escherichia*	trp	-	+	MNNG
W3110	*Escherichia*	polA$^+$	-	+	NG
p3478	*Escherichia*	polA$^-$	-	+	MNNG
D4	*Saccharomyces*	trp	NA	NA	MNNG

[a] An ampicillin sensitivity disc is placed on a fresh lawn of cells on complete medium. The zone of inhibition is compared with TA-98.

[b] A single drop of crystal violet solution is placed on a fresh lawn of cells on complete medium. The zone of inhibition is compared to a G-46 standard (resistant).

[c] Spot tests with reference mutagens are made to determine both correct mutant response and any contamination.

MNNG = N-Methyl-N'-nitro-N-nitrosoguanidine
QM = Quinacrine mustard
NF = Nitrofluorene
NG = Nitrosoguanidine

TABLE 2. BACTERIA AND YEAST STRAINS

Strain Designation	Gene Affected	Additional Mutations			References for Use in Screening
		Repair	LPS	R Factor	
TA-1535	*his* G	*uvr*^{B}	*rfa*	-	Ames et al. (1975)
TA-98	*his* D	*uvr*^{B}	*rfa*	pKM101	Ames et al. (1975)
TA-100	*his* G	*uvr*^{B}	*rfa*	pKM101	Ames et al. (1975)
W3110	-	polA$^+$	-	-	Slater et al. (1971)
p3478	-	polA$^-$	-	-	Slater et al. (1971)
D4	*ade2*, *trp5*	-	-	-	Zimmermann (1975)

TABLE 3. AMES ACTIVATION SYSTEM

	Component	MW	Supplier	Stock Preparation	Volume of Stock Added/ml of Final Mix	Final Concentration of Component/ml in Mix
1.	TPN	831	ICN	40 g/500 ml H_2O	40 µl	4 µmoles
2.	Glucose-6-phosphate disodium	282	Sigma	141 g/500 ml H_2O	5 µl	5 µmoles
3.	Sodium phosphate	142	Sigma	14.2 g/500 ml H_2O adjusted to pH 7.4	500 µl	100 µmoles
4.	$MgCl_2$	95	Sigma	19.2 g/500 ml H_2O	20 µl	8 µmoles
5.	KCL	76	Sigma	61 g/500 ml H_2O	20 µl	33 µmoles
6.	Homogenate	-	-	Standard KCL 9,000 x g supernatant	100 µl	Approximately 25 mg of fresh tissue equivalant
7.	H_2O				315 µl	

Components 1 and 2 are prepared in sterile distilled water and maintained at -20°C.

Components 3, 4, and 5 are prepared in distilled water, sterilized, and maintained at 4°C.

Components 6 is prepared and stored at -80°C until used.

Components 1-5 combined = core reaction mixture. MW = Molecular weight

Components 1-6 combined = complete S-9 mixture.

added to ensure reproducibility from sample to sample.

- Control Compounds: Unless otherwise specified, dimethylsulfoxide (DMSO) will be employed as the solvent for all test materials. DMSO has been carefully evaluated in the assay and has no mutagenic activity. The concentration of DMSO employed in the solvent control will be equal to the amount of DMSO added along with the highest concentration of test material and will likely not exceed 50 µl per plate.

QUALITY CONTROL -- Records of the manufacturer and lot number of DMSO employed will be maintained. Positive control compounds are listed in Table 4.

- Test Samples:

QUALITY CONTROL -- Upon receipt of the materials, the identifying designations and physical descriptions will be entered into a logbook and dated. All details of weighing and dilutions will be recorded.

Methods

- Preparation of Tissue Homogenates: Animals will be stunned, decapitated, and bled. The liver will be excised aseptically and placed in cold KCl. After washing with additional KCl, the tissue will be homogenized in 0.15 M KCl at a ratio of one part tissue to three parts KCl. The homogenate will be centrifuged at 9,000 x g for 10 minutes in a refrigerated Sorall centrifuge. The supernatant will be collected, pooled, and frozen at -80°C. Samples will be assigned a lot number and assayed for total protein and P-448 content.

QUALITY CONTROL -- The samples will also be checked for sterility.

- Plate Assay Method: Approximately 10^8 cells (0.1 ml) from an overnight culture of each indicator strain will be added to separate test tubes containing 2.0 ml of molten agar supplemented with biotin and a trace of histidine. For nonactivation tests the five dose levels of the test compound will be added to the contents of the appropriate tubes and poured over the surfaces of selected agar plates. In activation tests five dose levels of the test chemical will be added to the appropriate tubes with cells. Just prior to pouring, an aliquot of reaction mixture (0.5 ml containing the 9,000 x g liver homogenate) will be added to each of the activation overlay tubes, which will then be mixed, and the contents poured over the surface of a minimal agar plate and allowed to solidify. The plates will be incubated for 48 hours at 37°C, and scored for the number of colonies growing on each plate. Positive and solvent controls using both directly active positive chemicals and those that require metabolic activation will be run with each assay.

Dosing Procedures

- All types of chemicals can be evaluated with this technique. Solids, liquids, volatile liquids, and gases have all been screened through appropriate modifications of the procedure. Gases are tested by placing the plates and a measured amount of the gas in an airtight container of known volume for a fixed duration exposure.

Data Collection and Analysis

TABLE 4. POSITIVE CONTROL COMPOUNDS

Assay	Chemical	Solvent	Probable Mutagenic Specificity
Nonactivation	Methylnitrosoguanidine (MNNG)	Water or saline	BPS[b]
	2-Nitrofluorene (NF)	Dimethylsulfoxide[a]	FS[b]
	Quinacrine mustard (QM)	Water or saline	FS[b]
Activation	2-Anthramine (ANTH)	Dimethylsulfoxide[a]	BPS[b]
	2-Acetylaminofluorene (AAF)	Dimethylsulfoxide[a]	FS[b]
	8-Aminoquinoline (AMQ)	Dimethylsulfoxide[a]	FS[b]
	Dimethylnitrosamine	Saline	BPS[b]

[a] Previously shown to be nonmutagenic.

[b] BPS = Base-pair substitution.
FS = Frameshift.

- The raw data will be recorded on printed forms containing all relevant information concerning the test procedures.

QUALITY CONTROL -- All data sheets will be signed and dated by the responsible technician as the information is recorded. Copies of all raw data sheets will be attached to the final report. The standard deviation and standard error should be calculated for the solvent control plates from a minimum of 20 independent assays with each tester organism. Any data that fall outside the accepted range will be rejected. A complete set of evaluation criteria is to be provided with each final report.

3.4.2.5 Indicator organisms (tester strains)--

Considerable research has been conducted during the past two decades to develop sensitive and stable tester strains of microorganisms. The *Salmonella typhimurium* histidine auxotrophs are the best characterized and most widely used strains, particularly those developed by Ames and coworkers. Various *Escherichia coli* mutants, *Saccharomyces cerevisiae* strains, and *Neurospora crassa* mutants are also widely used (Table 3.4.4).

New strains are continually being developed and laboratories should keep in touch with leaders in the field for advice on strain selection. With reverse mutation systems, as many different strains as practicable should be used since each strain will detect mutagens which are able to induce a particular type of mutation only (Legator and Malling, 1971). Dosage of tester strains should be as great as possible to insure detection of compounds with low mutation rates. In the Ames test with *Salmonella typhimurium*, approximately 5 x 10^8 cells are usually used. In the host-mediated assay with *Salmonella*, *Neurospora*, or *Saccharomyces* strains, the animals are injected with about 6 x 10^8 cells.

All tester strains should be tested for original markers before starting a testing program and monitored frequently during use since some markers are somewhat unstable and can be lost. Ames strains should be routinely checked for 1) histidine requirement, 2) deep rough (RFA) characteristic, 3) R factor (TA 98 and TA 100), UvrB deletion, and 5) spontaneous reversion rate.

Spontaneous mutation (reversion) rates on control plates without mutagen and S-9 mix after 48 hours for Ames strains are reported to be as follows (Ames et al., 1975):

Strain	Spontaneous mutants/plate
TA 100	160
TA 98	40
TA 1538	25
TA 1535	20
TA 1537	7

Rates are slightly higher on plates with S-9 mix. The normal bacterial spontaneous mutant frequency ranges from 1 x 10^{-9} to 1 x 10^{-11} ([illegible] et al., 1972).

TABLE 3.4.4 SOME MICROBIAL INDICATOR STRAINS AVAILABLE FOR MUTAGENICITY ASSAYS (Brusick et al. 1976)

Organism	Strain	Probable (*) Event Detected
Salmonella typhimurium	G-46	R-BPS
	TA-1530	R-BPS
	TA-1535	R-BPS
	TA-1536	R-FS
	TA-1537	R-FS
	TA-1538	R-FS
	TA-100	R-BPS
	TA-98	R-FS
Escherichia coli	WP_2	R-BPS
	WP_2uvrA^-	R-BPS
	K12/343/113	FM
	CM_{561}	R-BPS
	CM_{661}	R-BPS
	W3110/P3478	R
Saccharomyces cerevisiae	S288Ca	FM
	S211	R-BPS
	S138	R-FS
	D3	MR
	D4	MGC
	D5	MR
	D5	MR
	S288C/774-6A	ER

---

* R - Reverse; BPS - Base-pair substitution mutation; FS - Frameshift mutation; FM - Forward mutation; MR - Mitotic recombination MGC - Mitotic gene conversion; ER - Excision repair

3.4.2.6 Controls--

Positive controls as well as negative controls (including vehicle controls) must be included in every test. Moreover, the positive compound should be chemically similar to the test chemical. Positive controls used by one commercial laboratory are shown in Table 3.4.5. In Salmonella assays, dimethylnitrosamine is usually given in the form of a single 50 mg/kg oral dose. Ethylmethanesulfonate is generally administered intramuscularly at a level of 350 mg/kg in Saccharomyces assays (Green et al., 1976).

TABLE 3.4.5 POSITIVE CONTROLS USED IN NONACTIVATION AND ACTIVATION ASSAYS (Brusick et al., 1976)

Assay	Chemical	Solvent	Probable Mutagenic Specificity
Nonactivation	Ethylmethanesulfonate (EMS)	Water or saline	BPS*
	Methylnitrosoguanidine (MNNG)	Water or saline	BPS*
	2-Nitrofluorene (NF)	Dimethylsulfoxide**	FS*
	Quinacrine mustard (QM)	Water or saline	FS*
Activation	2-Anthramine (ANTH)	Dimethylsulfoxide**	BPS*
	2-Acetylaminofluorene	Dimethylsulfoxide**	FS*
	8-Aminoquinoline (AMQ)	Dimethylsulfoxide**	FS*
	Dimethylnitrosamine (DMNA)	Saline	BPS*

* BPS = Base-Pair Substitution
FS = Frameshift

** Previously shown to be nonmutagenic

3.4.2.7 Proficiency Testing--

In spite of the fact that positive as well as negative controls are usually included in mutagenicity tests, proficiency testing of unknowns should be an integral part of the quality control program in mutagenicity testing. The use of unannounced unknowns which can be slipped into the daily flow of work together with routine testing slips should give the best indication of the laboratory's proficiency in the area. Interlaboratory cooperation with new chemicals is advisable also.

3.4.3 Microorganisms - General Toxicity Testing

Numerous attempts have been made to develop microbial assays to replace the costly and time-consuming animal tests for general toxicity testing of chemicals and other substances. The greatest achievement to date in this area appears to be the *Ciliastasis* assay using ciliated protozoa (Woodard, 1976). Cilia of human respiratory tract epithelium keep the respiratory system free from foreign matter by sweeping a blanket of mucus toward the esophagus at the upper end of the tract. The cilia of several species of *Paramecium* and *Tetrahymena* have been found to respond to components of cigarette smoke in a manner quite similar to the response of human tracheal cilia. The ciliastasis assay using these test species is regarded by some workers as having predictive value for effects of smoke and other pollutants on the human respiratory tract (Ballenger, 1960; Wang, 1963; Kensler and Battista, 1963; Weiss and Weiss, 1964; Wynder and Hoffman, 1964; Weiss, 1965; Kennedy and Elliott, 1970).

3.4.3.1 Methods--

Early toxicity studies with protozoa used growth inhibition as the endpoint. Protozoa are similar to both undifferentiated prokaryotic microorganisms such as bacteria and the more complicated metazoans in many respects and have been regarded as bridging the gap between the two groups. Although protozoa are unicellular organisms they have highly developed and specialized organelles for locomotion and reproduction. Metabolic and nutritional requirements are even similar to those of mammalian cells (Woodard, 1976). Their growth in pure culture has been compared to the growth of somatic tissue cells in multicellular organisms (Jacob, 1958).

Some of the assays that have been in use recently are outlined in Table 3.4.6.

Although the ciliastasis assay with *Tetrahymena* and *Paramecium* has been developed mainly in connection with cigarette smoke toxicity studies, the test is also applicable for air pollutants, stack emissions, and environmental contaminants contained in soil and water.

3.4.3.2 Experimental Design--

Dalhamn and Rylander in 1969 made a critical study of methodologies employed in the toxicologic evaluation of tobacco smoke on the respiratory system and developed the following guidelines for smoke toxicity experiments:

- The smoke should be analyzed and the concentration of particulates be determined.
- Exposure of the test organisms should be comparable to human exposure during smoking with respect to the amount of smoke and duration of exposure. One 35-ml puff drawn for 2 seconds once every minute was found to be the smoking method most widely used.
- Smoke should be tested as a suspension in air rather than as a solution or condensate.

  In humans, an average of approximately 35 ml of smoke is inhaled into the lungs along with about 200 to 300 ml of fresh air. Exposure is short in the upper respiratory tract but is considerably greater deep down in the lungs.

TABLE 3.4.6 TETRAHYMENA AND PARAMECIUM ASSAYS

Strain Used	Toxic Substance	Experimenter
Tetrahymena pyriformis (Strain E)	Aminoazobenzene Monomethylaminoazobenzene Dimethylaminoazobenzene 3'-Methyl-4-dimethylaminoazobenzene Methyl red 8-Azaguanine	Jacob (1958)
Tetrahymena p.	Cigarette smoke	Kennedy and Elliott (1970)
Tetrahymena p.	Nontobacco smoke Lettuce *Poa pratensis*	Gray and Kennedy (1974)
Paramecium caudatum	Benzo(a)pyrene	Epstein et al. (1963)
Paramecium aurelia (Strain 51)	Tobacco smoke Tobacco leachate Tobacco ash Cigarette paper ash	Wang (1963)
Paramecium a.	Cigarette smoke	Weiss and Weiss (1964, 1967) Weiss (1965, 1968)

- Experiments should be of long duration and should correspond to a lifetime exposure situation in humans.

- In vivo systems are preferred although in vitro assays are acceptable for screening studies. Animal species most closely related to man should be employed.

3.4.3.3 Quality Control--

All lots of culture media and reagents should be tested for performance upon receipt and at regular intervals thereafter. Careful attention should be paid to the manufacturer's storage recommendations and expiration date. All sterility tests should be conducted in replicate inside a biological safety cabinet with adequate environmental air controls.

Negative and positive controls should be included in each test. Negative controls should include reagents, vehicle, etc., as well as untreated cultures.

Positive control cultures should be treated with a substance known to be toxic to the tester strain which is of the same chemical class as the test chemical.

All assays should be replicated whenever possible with different samples and on different days with different lots of the tester organism.

The tester strains employed by the authors of an assay procedure should always be used unless alternate strains have been specified as being acceptable. The preparation of large lyophilized pools of the test organism is advisable to eliminate variation in this component of the assay system.

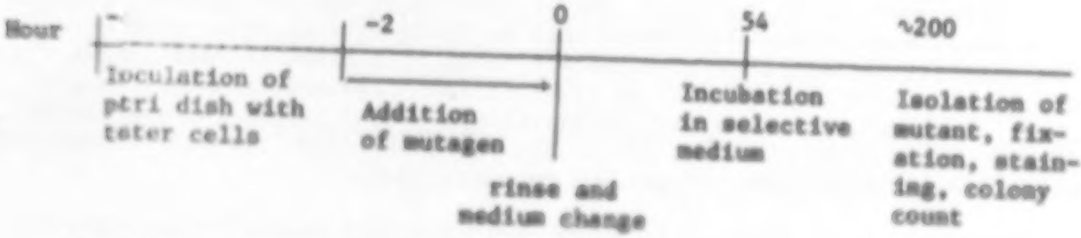
Laboratories engaged in a routine large-scale screening operation may profit from a proficiency test program to identify shortcomings and problem areas. Unknowns can be slipped into the daily samples from time to time and results used as the basis for corrective measures. Cooperative interlaboratory tests are also valuable in this respect.

All precision equipment employed in this assay such as incubators, balances, etc., should be cared for as outlined in the manufacturer's maintenance warranty requirements. Instruments should be inspected regularly and recalibrated at intervals and by procedures recommended by the manufacturer.

A quality control record book should be maintained for recording all quality control operations. All entries should be signed by the responsible personnel involved.

3.4.4 Cell Cultures - Mutagenicity Testing

The applicability of mammalian cells in culture for mutagenicity testing was discovered independently by Chu and Malling (1968) and by Kao and Puck (1968). Both groups used essentially the same general experimental procedure:


Hour
-
-2
0
54
~200
Inoculation of petri dish with tester cells
Addition of mutagen
rinse and medium change
Incubation in selective medium
Isolation of mutant, fixation, staining, colony count

Since these initial discoveries, a number of mutagenicity assays employing somatic and germinal mammalian cell cultures, including human cells, have been developed. Since mammalian cells differ from those of lower organisms in metabolism, DNA repair processes, and a more exact translation of the genetic code, the use of a mammalian system for mutagenicity testing has advantages (Legator, 1972). Mammalian cell genetics bridges the gap between mutational studies in microorganisms and those in the intact animal (Chu, 1971). Phenotypic markers which may be used in gene mutation tests include nutritional, biochemical, and serologic factors, drug resistance, temperature sensitivity, and radiation sensitivity (Chu, 1971; Anon., 1975). The use of human cells is limited somewhat due to the fact that they are genetically stable in vitro for only about 50 passages and have a low plating efficiency (about 10%). However, the field of mammalian genetics is relatively young and methods for overcoming these technical difficulties may be forthcoming soon. The use of nondividing primary human lymphocytes (S stage) has already circumvented some of these difficulties. Moreover, there is no good reason for believing that mutational effects in other mammalian cells are not the same as those in human cells (Chu, 1971).

3.4.4.1 Cell Identification and Monitoring--

In general, mammalian cells used for mutagenicity testing should have the following minimal characteristics:

- High sensitivity
- High plating efficiency
- Stable karyotype
- Low spontaneous mutation rate
- Response to metabolic activation
- Absence of mycoplasma and virus

All cell cultures should be subjected to identification tests upon receipt and at regular intervals during use. Many cell lines look alike and can be identified only by specialized tests. Incorrect identification can result from mislabelling by the manufacturer or by contamination during preparation or in the user's laboratory. Cell cultures can be identified by the cytotoxic antibody test or by the use of the fluorescent-labelled antibody test; both tests require cell-specific antiserum which is available commercially.

In the cytotoxic antibody test, samples of the cell suspension (1×10^5 to 2×10^5) are incubated with specific diagnostic antisera at 37°C for one hour. Complement or guinea pig serum is then added and the mixture incubated for another 30 minutes. Complement will be fixed only by cells combined with their specific antibody; these cells will then be killed. A small amount (0.1 ml) of trypan blue (0.25%) is added to each tube and the cells are examined in a hemocytometer under the microscope. Blue cells are dead; living cells are white. If more than 50% of the cells are dead, the test is positive for the antiserum present (Greene and Charney, 1973).

The fluorescent-labelled antibody test is a very sensitive procedure for cell identification and can also detect contaminants at levels as low as 1/10,000 cells. Cell samples are treated with specific labelled antibodies covering the range suspected and then examined under the fluorescent microscope. Cells will combine only with their specific antibody in significant numbers. Cell-antibody combinations show an "apple-green" fluorescence under the scope since the antibodies used are labelled with fluorescein isothiocyanate. The cells are identified, as in the preceding test, by the specific antibody with which they have combined (Stulberg and Simpson, 1973).

Mammalian cell cultures sometimes undergo genetic changes - loss of markers, change in number or character of chromosomes, etc. - and must be monitored frequently for genetic stability. Karyotype analysis is also of value for identification. Log phase cultures are treated with colcemid or velban (0.06 µg/ml) for 2 hours at 37°C. After rinsing, the culture is treated with trypsin or pronase to separate cells. The suspension is then washed and transferred to a hypotonic medium (medium and H_2O, 1:2). The cells are next collected by centrifugation and fixed with methyl alcohol and glacial acetic acid (3:1). Thin suspensions on slides are then stained with acetic-orcein, rinsed, air-dried and mounted for chromosome analysis. Various banding techniques have also been developed for genetic analysis (Hsu, 1973). Preparation of a large number of single-test ampoules of cells for storage in liquid nitrogen (-196°C) will greatly reduce the amount of monitoring necessary (U.S. EPA, 1977). A complete history of each cell line or strain used in mutagenicity testing as well as all monitoring results (identity, karyotype, mycoplasma, viruses, etc.) should be recorded in the quality control record book.

Cell cultures must also be monitored frequently for the presence of mycoplasma (PPLO) organisms. Mycoplasmas are bacteria-like microorganisms which lack cell walls and hence are very pleomorphic. The smallest reproductive units (100-125 nm) are as small as medium-sized viruses and readily pass through many filters. Several species are members of the normal flora of mouth and genitourinary tract. Many continuous cell lines are parasitized by these agents which, unfortunately, usually give no evidence of their presence (Clive et al., 1973; Hayflick, 1973; Barile, 1973).

The three main sources of mycoplasma contaminants are personnel (mouth pipetting), serum (bovines), and typsin (swine). With the advent of mechanical pipettes, contaminants from workers have decreased greatly. Today more than 50% of the contamination comes from serum. Serum should be inactivated at 56°C for 30 minutes and then filtered twice through a 220 nm filter; trypsin is usually filtered through a 100 nm filter (Barile, 1973).

Mycoplasmas can be isolated in Edward-Hayflick broth or in the semisolid broth (SSB) medium of Barile (Barile, 1973), although samples as large as 25-100 ml may be necessary. The broth cultures are usually streaked over an agar medium for the formation of colonies with the characteristic "fried egg" appearance. The colonies are very small and require 50-100 x magnification for detection. Moreover, various artifacts - pseudocolonies (Ca and Mg soaps), air bubbles, clumps of tissue cells, and condensed water - make detection difficult.

Monitoring cell cultures and reagents (serum and trypsin) for mycoplasmas requires personnel with specialized training and laboratories not so staffed should engage the services of a mycoplasmatologist for this work.

Elimination of mycoplasmas from contaminated cell cultures is very difficult. According to Barile (1973) the best method consists of a combination of a high-titered antiserum (3% final concentration) and tetracycline (10 µg/ml) or kanamycin (100 µg/ml) in the growth medium. However, if the contaminated cell type is commercially available, it may be more economical from the standpoint of time and money to purchase a new stock from a reliable manufacturer who supplies certified mycoplasma-free cultures.

In addition to the "mycoplasma menace", cell cultures may carry "passenger" viruses without any evidence of their presence. Many human lymphoblast cultures have been found to contain the Epstein-Barr virus (Sato et al., 1972). Uncertified chick embryo cultures usually contain avian leukosis virus and the SV_{40} virus may be present in several types of monkey kidney tissue cultures (Jawetz et al., 1972). Since some viruses cause chromosome aberrations and other genetic effects, mutagenicity test results with such cultures may be grossly erroneous, and are always suspect.

Discovery of mycoplasmas, viruses, or any other contaminants in tester strains requires, of course, complete re-assay of all mutagenicity test compounds back to the last previous certification that the cultures were "clean".

3.4.4.2 Cell Population--

The number of cells employed in a mutagenicity assay should be sufficient to detect mutation at double the spontaneous mutation frequency, at least, of the tester strain (U.S. EPA, 1977). In general, sensitivity is directly proportional to the number of cells used. However, several workers have found that increasing the cell population often decreases the mutation frequency. Chu and Malling (1968), for example, found that increasing the cell inoculum from 2.5×10^5 to 10×10^5 decreased the mutation frequency from 11.3 to $0.4/10^5$ survivors, in assays using Chinese hamster V-79 cells to detect mutation at the glutamine (gln) and 8-azaguanine (azg) loci. Shapiro et al. (1972) discovered that the "concentration effect" varied with the cell type. Increasing the concentration of Chinese hamster cells from 10^4 through 10^5 to 10^6 decreased the mutation frequency.

With certain human cell lines (L-53 and L-54) increase of the cell population at the same rate (10^4 to 10^6) caused an increase in the mutation frequency. In all mutagenicity tests, the plating population to be used with the selective medium which is optimal for the survival of mutants must be determined in preliminary tests before assays are conducted (Shapiro et al., 1972). In general, any mutagenicity assay system should be calibrated with known positive and negative mutagens before testing is begun.

3.4.4.3 Dosage of Test Chemical

Two of the most widely used cell culture assays are Unscheduled DNA Synthesis (with and without microsomal activation) and the host-mediated assay using mouse lymphoma L5178Y cells (Anon. 1975; U.S. EPA, 1977; de Serres, 1974; Legator, 1976).

Dosage varies considerably in unscheduled DNA synthesis assays as well as in the host-mediated assay. In both types of test the determining factor is mainly toxicity of the chemical (Lieberman et al., 1971; Stich and San, 1970; Clarkson and Evans, 1972). A wide range of doses should be used. The maximum dose must cause some toxic effect and should be sufficiently large to detect weakly-acting mutagens; at least four lower doses, appropriately spaced, should be included (U.S. EPA, 1977). If toxicity of the compound is not known, the dosage range may be based upon results of a preliminary toxicity test (Brusick et al., 1976). The tester cultures should be exposed to action of the chemical (dosing period) for at least one hour (U.S. EPA, 1977). It is important to test technical grades and formulations as well as purified specimens in the case of commercial chemicals (U.S. EPA, 1977). Typical dosage ranges reported in recent assays by various workers are shown in Table 3.4.7.

In the host-mediated assay using mouse lymphoma L5178Y asn^{-} cells in BDF male mice, Capizzi et al. (1973) obtained 39 mutants per 10^6 cells with sulfur mustard at a dosage 100 mg/kg. In vitro, the chemical-induced mutation occurred at a dosage as low as 0.001 μg/ml.

3.4.4.4 Methods--

- Unscheduled DNA Synthesis

The unscheduled DNA synthesis mutagenicity test is based upon the assumption that mutagens damage DNA and that this effect can be detected by the incorporation of DNA precursors into the DNA of nondividing cells (Stoltz et al., 1974). Unrepaired or misrepaired DNA damage will result in gene mutations or other changes which affect gene function (U.S. EPA, 1977).

A variety of mammalian cell cultures, including Syrian hamster, Chinese hamster, normal human cell strains (WI-38, etc.) as well as neoplastic human cell lines (HeLa, etc.) have been used in unscheduled DNA synthesis assays (Brusick et al., 1976; Painter and Cleaver, 1969; Stich and San, 1970). Primary peripheral human lymphocytes have also been used by some workers (de Serres, 1974; Lieberman et al., 1971; Clarkson and Evans, 1972). Standardized human cell strains from reliable repositories are recommended (U.S. EPA, 1977).

TABLE 3.4.7 INDUCTION OF UNSCHEDULED DNA SYNTHESIS BY VARIOUS COMPOUNDS IN VITRO: DOSAGE RANGE

Chemical	Dosage Range	Exposure Period	Cell Type	Reference
4-Nitroquinoline 1-oxide	1×10^{-5} to^{-}5 $\times 10^{-8}$ M	1.5 hr.	Human; Chinese hamster	Stich and San, 1970
Nitrogen mustard (NH_2) Ethylmethanesulfonate (EMS) Methylmethanesulfonate (MMS)	10^{-1} to 10^{-7} M	1.0 hr.	Human lymphocytes	Lieberman et al., 1971
Nitrogen mustard (NH_2)	10^{-4} to 10^{-5} M	1.0 hr.	Human lymphocytes	Clarkson and Evans, 1972

A protocol for Unscheduled DNA Synthesis (UDS) in Human WI-38 Cells is given in the following pages.

EXAMPLE: UNSCHEDULED DNA SYNTHESIS (UDS) IN HUMAN WI-38 CELLS

Purpose of Study

- Mutagenicity determination

Design of Experiment

- Cell Cultures: Normal human diploid WI-38 cells are seeded at 250,000 cells in 60 mm tissue culture dishes. The cells are grown to confluency in Eagle's Minimum Essential Medium (MEM) plus 10% fetal calf serum (FCS). They are then kept in MEM containing 0.5% FCS for 5 days.

- Dosage of Test Substance: Dosages are determined from preliminary toxicity curves established from treatment with 1.0, 0.1, 0.01, and 0.001% levels of the test substances; three dose levels of each substance are selected for mutagenicity testing.

- Controls: Positive and negative (solvent and untreated) controls are included in each test.

Conduct of Experiment

- Nonactivation Assay: Nonproliferating cultures, arrested in G_1 phase of the cell cycle by contact inhibition and 0.5% FCS synchronization medium, are exposed to three doses of the test substance determined as indicated above. Tritiated thymidine is added to the cultures along with the test substance. In order to prevent any scheduled DNA synthesis from taking place, hydroxyurea is added to the cultures one hour before addition of the test substance and is included in the medium at each change. Exposure to both test substance and radioactive label is terminated by washing the cells in Hanks' Balanced Salt Solution that contains an excess of unlabelled thymidine.

QUALITY CONTROL -- The entire mutagenicity assay system should be calibrated with known positive and negative mutagens and promutagens before routine testing is begun.

The cell cultures must be free from mycoplasma contaminants which are capable of incorporating radioactive thymidine in addition to causing other effects which invalidate assays.

Conduct of Experiment

- Activation Assay: The activation assay is identical to the noninactivation assay except that an aliquot of 100,000 g liver microsomal extract containing the following components is included in the incubation of cells with the test chemical:

Component	Final Conc./ml
TPN (sodium salt)	6 μmoles
Isocitric acid	35 μmoles
Tris buffer, pH 7.4	28 μmoles
$MgCl_2$	2 μmoles
Liver extract equivalent to 25 mg fresh tissue	

QUALITY CONTROL -- Donor animals for the microsomal extract should be pre-induced with a compound known to be effective for the class of substances to be tested.

- Radioactivity incorporated by cells during exposure to test substance and radioactive thymidine indicates unscheduled DNA synthesis and hence DNA repair.

QUALITY CONTROL -- Glassware, pipettes, water, media, and reagents must be of tissue culture grade. Media and reagents (including tritiated thymidine) must be performance-tested before use.

- Amount of tritiated thymidine incorporated into DNA during repair is determined by solubilizing the cells and extracting the DNA.

- One aliquot of the DNA extracted is processed for determination of the amount of radioactivity by scintillation counting.

QUALITY CONTROL -- All tissue culture work should be performed in laminar flow cabinets equipped with HEPA filters to safeguard against airborne microbial contaminants.

- A second portion is used to determine spectrophotometrically the amount of diphenylamine-reactive DNA.

Results are expressed as radioactivity (DPM) per milligram of DNA.

In unscheduled DNA Synthesis Assays it is essential that the cell cultures be maintained in the nondividing s-state since incorporation of tritiated thymidine in scheduled DNA synthesis during mitosis would overshadow that involved in repair of the mutagen-damaged DNA (Anon., 1975). In assays with peripheral human lymphocytes, hydroxyurea (HU) is used to keep the occasional cell not in the s-state from replicating (Lieberman et al., 1971). Arginine-deprivation has been used by Stich and others to prevent replicative DNA synthesis in a variety of other cell types (Stich and San, 1970; Stich et al., 1971). It is essential that the laboratory assess the system employed before starting mutagenicity testing to make certain that replicative DNA synthesis does not occur in the test system.

Incorporation of tritiated thymidine (^{3}H-TdR) by nondividing cells is usually used as the indicator of DNA repair of mutagen-induced damage (Anon., 1975; U.S. EPA, 1977; Lieberman et al., 1971; Stich and San, 1970). The labelled reagent may be added with the test compound in the continuous method or pulsed for an appropriate period only. Incorporation of ^{3}H-TdR is measured by autoradiography or by scintillation counting using standard techniques (Stich and San, 1970; Stich et al., 1971; Lieberman et al., 1971).

Acceptance specifications of each lot of tritiated thymidine should be carefully checked upon receipt. Acceptable lots must also be performance tested with both positive and negative standards. Source, lot number, purity, concentration, and specific activity of all preparations used should be included in the mutagenicity test report (U.S. EPA, 1977).

- Host-mediated Assay

The host-mediated assay with mouse lymphoma cells (L5178Y) is the second major mammalian in vitro cell test widely recommended for mutagenicity testing. The L5178Y strain has been used extensively for a number of years in a variety of studies and is very well characterized. It has an essentially diploid karyotype, a high plating efficiency, and grows well in vitro (Anon., 1975; U.S. EPA, 1977; de Serres, 1974; Legator, 1976) Tumorgenicity is not regarded as a drawback in mutagenicity testing since mutagenic mechanisms of tumor cells and normal cells are believed to be essentially the same. Moreover, the induced thymidine kinase (TD) mutation rate with L5178Y was found to be essentially the same as in other mammalian cells in cultures (de Serres, 1974).

A protocol for the Mouse Lymphoma Forward Mutation Assay is given in the following pages.

EXAMPLE: MOUSE LYMPHOMA FORWARD MUTATION ASSAY

Purpose of Study

- Mutagenicity determination

Design of Experiment

- Indicator Cells: L5178Y Thymidine kinase ($TK^{+}/^{-}$) mouse lymphoma cells are used. The cells are heterozygous for a specific autoxomal mutation at the TK locus and are bromodeoxyuridine (BUdR)-sensitive. Scoring for mutation is based on selecting cells that have undergone forward mutation from a $TK^{+}/^{-}$ to a $TK^{-}/^{-}$ genotype through the use of BUdR-supplemented soft cloning agar.

- Media: Maintenance medium - Fischer's mouse leukemia medium with 10% horse serum and sodium pyruvate.

Cloning medium - Fischer's medium with 20% horse serum, sodium pyruvate, and 0.37% agar.

Selection medium - Cloning medium plus 0.5 mg of BUdR/100 ml.

- Dosing Procedure: All types of chemicals can be evaluated in the mouse lymphoma assay. Solids are dissolved in suitable solvents and added to test system at seven predetermined levels. Liquids are added directly to the cultures at seven concentrations or following dilution in appropriate solvents. Highly volatile liquids (vapor phase test required) are added at seven dosages to an air-tight container of fixed volume and allowed to completely volatilize in presence of the exposed cell population. Known volumes of gases are measured into an air-tight container of fixed volume. The volume of gas is graded at seven dose levels.

For most chemicals, seven concentrations are selected for assay on the basis of a preliminary toxicity test. At least four of the dosages are then cloned for the mutagenicity evaluation.

- Vehicle: Tissue culture growth medium or dimethylsulfoxide (at 1.0%, or lower, final concentration) are used as solvents for the test substances.

- Controls

Positive control: Ethylmethanesulfonate (200 µg/ml), which induces mutation by base-pair substitution, is used for nonactivation tests. Dimethylnitrosamine (500 µg/ml, which requires metabolic biotransformation by microsomal enzymes to induce mutation, is employed in the activation test.

Negative control: The solvent in which the test substance is dissolved serves as the negative control.

Microsomal activation system

Male randon-bred mice are used as the source of hepatic microsomes. The mice are killed by cranial blow, decapitated, and bled. The liver is immediately dissected from the animal using aseptic technique and placed in ice cold 0.25 M sucrose buffered with Tris buffer at pH 7.4. When an adequate number of livers has been collected, they are washed twice with fresh buffered sucrose and completely homogenized. The homogenate is centrifuged for 10 minutes at 9,000 x g in a refrigerated centrifuge. The supernatant from this centrifuged sample is retained and frozen at -80°C until used in the activation system. This microsome preparation is added to a "core" reaction mixture to form the activation system described below:

Component	Final Concentration/ml
1. TPN (sodium salt)	6 µmoles
2. Isocitric acid	35 µmoles
3. Tris buffer, pH 7.4	28 µmoles
4. $MgCl_2$	2 µmoles
5. Homogenate fraction equivalent to 25 mg of wet tissue	

SUMMARY OF TESTS INCLUDED IN THE L5178Y MOUSE LYMPHOMA ASSAY

Test	L5178Y	
	Trail A	Trial B
	Nonactivation	Activation
1. Solvent Control	X	X

SUMMARY (Continued)

Test	L5178Y Trial A Nonactivation	L5178Y Trial B Activation
2. Positive Control		
Ethylmethanesulfonate	X	
Dimethylnitrosamine		X
3. Test Chemical		
Dose Level		
1	X	X
2	X	X
3	X	X
4	X	X

Conduct of Experiment

- Nonactivation Assay: A modification of the procedure of Clive and Spector (1975) is used. Prior to treatment with the test substance, the indicator cells are cleaned of spontaneous $TK^{-}/^{-}$ mutants by growing them in a medium containing thymidine, hypoxanthine, methotrexate, and glycine (THMG). This medium permits survival of only those cells that produce the enzyme thymidine kinase and can therefore utilize exogenous thymidine from the medium. The cleansed cells are exposed to the test substance (solid, liquid, volatile liquid, or gas) at predetermined doses for five hours. The treated cells are then washed, fed, and allowed to express in growth medium for 3 days. Daily counts are made.

QUALITY CONTROL -- The assay system should be calibrated with known positive and negative mutagens and promutagens before routine testing is begun. Indicator cells should be free from *Mycoplasma* contaminants and "passenger" viruses.

- Activation Assay: The activation assay differs from the nonactivation test in the following manner only. Two ml of the activation mixture is added to 10 ml of growth medium. The desired number of cleansed cells is then added to this mixture and the flask incubated on a rotary shaker for five hours. The incubation is terminated by washing the cells twice with growth medium. The washed, treated cells are then allowed to express for three days as described above for the nonactivation assay.

QUALITY CONTROL -- Donor animals for microsomal extract should be pre-induced with a compound known to be effective for the class of substances to be tested.

Observations and Tests

- At the end of the three day expression period described above, $TK^{-}/^{-}$ mutants are detected by cloning the cells in the selection medium for ten days. The surviving cell population is determined by plating diluted aliquots in nonselective growth medium.

- A mutation index is derived by dividing the number of clones formed in the BUdR-containing selective medium by the number found in the same medium without BUdR. The ratio is then compared to that obtained from the other dose levels and from positive and negative controls. Compound-related toxicity based on cell growth in suspension and cloning efficiencies are also included in the final report.

3.4.4.5 Quality Control

- Control Preparations

The laboratory should assess the sensitivity and reproducibility of all assay systems to be used, with appropriate positive and negative controls, prior to the beginning of mutagenicity testing. Interlaboratory testing is strongly recommended to determine reproducibility of the testing procedures. In each individual assay, the laboratory must include positive controls of the same chemical class as the test compound as well as vehicle and untreated controls (U.S. EPA, 1977).

- Tissue Culture Glassware, Media, Reagents, etc.

Mammalian cells growing in vitro are usually quite delicate and very fastidious with respect to nutrients. Scrupulously clean glassware as well as properly-formulated media are required for satisfactory results.

Most laboratories today use disposable polystyrene plastic or soda glass containers. Plastic containers may be used as packaged although laboratories should be careful to use the brand specified by author of the method since some brands have been found to be toxic. Soda glass containers must be washed or rinsed before use. Most items are machine-washed with special detergents and rinsed at least 12 times since very minute amounts of detergents or cleaning compounds are toxic; the final rinses should be with demineralized water. The best quality borosilicate glass must be used for storage of cells or for continuous cultures carried in the laboratory (Coriell, 1973b). All lots of containers, pipettes, syringes, etc., should be tested in replicate with the cell type to be used prior to mutagenicity testing.

The water used in tissue culture work must be of the highest purity. Conductivity should be in the range of 1 to 2 x 10^6 ohms. The use of a mixed bed ion exchanger followed by glass distillation is regarded as the best procedure. Teflon or borosilicate glass carboys are preferable for storage although water for tissue culture purposes should not be stored for long periods of time at room temperature. Great care must be taken to avoid contamination by pyrogens. Some laboratories store batch lots of water in the refrigerator (Pumper, 1973).

Many laboratories use commercial tissue culture media which require only the addition of the usual supplements such as serum, glutamine, etc. These basal media from reputable manufacturers are usually of very high quality and require monitoring only infrequently. Supplements and special reagents, however, require strict monitoring. In a one-year survey (1968-1969) of fetal calf serum, 10% of all lots were contaminated with bovine viruses, bacteria and mycoplasmas (Boone, 1973). In another survey, trypsin accounted for almost 20% of all mycoplasma contamination (Barile, 1973).

All tissue culture work should be performed in laminar flow cabinets equipped with HEPA filters. Masks should be worn and the hair covered. Talking should be reduced to a minimum and gum-chewing is taboo. Scrupulous personal hygiene should be encouraged. Floor, walls, and work surfaces should be scrubbed with disinfectant prior to work. In addition to these elaborate precautions, a detailed performance and sterility testing program, covering all media, reagents, and materials, should be instituted. All instruments and other equipment should be recalibrated at stated intervals and checked frequently. Freezers should be equipped with an alarm system. All quality control data should be entered into a bound notebook, dated, and signed by responsible personnel.

3.4.5 Cell Cultures - Carcinogenicity Testing

It is estimated that there are at present approximately two million known chemical compounds, and over 30,000 of these are now in commerce. Our daily exposure to many of these chemicals in food, water, cosmetics, and in the environment has caused increasing concern over their possible carcinogenic and/or other toxic effects (Anon., 1973). A heroic attempt is being made to evaluate this hazard through definitive tests in mammals but the burden is too great from the standpoint of time, money, and personnel. It is becoming increasingly apparent that rapid, sensitive, and reliable in vitro methods are needed to screen the large number of new chemicals created each year in addition to the huge backlog of untested compounds to which society is now exposed (Woodard, 1976). A concerted effort is being made in this direction and Stich et al. (1975) in a recent survey of the problem list no less than 26 bioassays which show promise for the detection of chemical carcinogens (Table 3.4.8). Of this array, four are generally regarded as being the most promising for screening purposes (Stoltz et al., 1974; Stich et al., 1975):

- Cell transformation
- Unscheduled DNA synthesis (DNA repair synthesis)
- Ames Salmonella test
- Drosophila melanogaster recessive mutation test

Although transformation is the only recommended test which is directly related to the carcinogenic process, all have given good results in the detection of chemical carcinogens (Anon., 1973; Stoltz et al., 1974). However, it should be noted that none of these tests at this time will absolutely identify a carcinogen. Their main value at present is to provide a rapid main screening system to detect "high risk potent carcinogens" and thus greatly reduce the animal assay load in definitive carcinogenicity testing (Anon 1973). At present, all positive cell transformation tests must be confirmed by production of cancer in animals following the injection of the transformed cells. Cell transformation, at this time, is thus an in vitro-in vivo assay procedure (Anon., 1971).

TABLE 3.4.8 PROMISING BIOASSAYS FOR THE DETECTION OF CHEMICAL CARCINOGENS (Stich et al., 1975)

Test Species	Effect	Reference
MOLECULES		
- B. subtilis, D. pneumoniae H. influenza, H. streptococci	Genetic transformation	Herriott, 1971; Maher et al., 1970
- DNA	Flourescence	Morgan and Pulleyblank, 1974
VIRUSES		
- E. coli K-12(λ)	Phage induction	Heinemann, 1971
MICROORGANISMS		
- Salmonella typhimurium	Frame shift mutations, base pair substitution,	Ames et al. 1973a, b
- B. subtilis	"rec" assay	Kada et al., 1972
- E. coli (exc^{-}, polA^{-}, rec^{-})	Differential killing, Mutations	Slater et al., 1971 Ishii and Konod, 1975
EUKARYOTES		
- Tetrahymena pyriformis	Unequal division	Moutan and Fromageot, 1971 Mita et al., 1969
- Saccharomyces cerevisiae	Mutations, gene conversion Mitotic crossing over UV^{-}, rec^{-}, differential killing	Zimmermann, 1975 Koske and Stich, 1973 Fahrig, 1974
- Neurospora crassa	Mutations, and -3 region	De Serres, 1974
- Aspergillus nidolans	Nondisjunction Crossing over	Bignami et al., 1974
PLANTS		
- Vicia, Pisum, Allium	Chromosome aberrations	Kihlman, 1966
- Hordeum vulgare	Chromosome aberrations	Wuu and Grant, 1966
INVERTEBRATES		
- Drosophila melanogaster	Mutations	Sobel, 1974; Vogel, 1971
- Bombyx mori		Tazima and Onimaru, 1974

Continued

TABLE 3.4.8 (Continued)

Test Species	Effect	Reference
MAMMALS		
- Bone marrow (rodents)	Chromosome aberrations	Barthelmess, 1970
	Micronucleus test	Schmid, 1975; Heddle, 1973
- Cultured cells	Chromosome aberrations	Barthelmess, 1970
	Mutations	Chu, 1972; Clive, 1974
	DNA fragmentation	Laishes and Stich, 1973a,b
	DNA repair	Stich et al., 1972a San and Stich, 1975
	Transformation	Kuroki, 1974; Heidelberger, 1973 Di Paolo et al., 1969
- Sperm	Morphological anomalies	Wyrobek et al., 1975
MAN		
- Peripheral lymphocytes	Chromosome aberrations	Evans and O'Riordan, 1975
	Micronucleus test	Heddle, 1973; Schmid, 1975
- Lymphocyte cultures - Cultured cells	Chromosome aberrations	Barthelmess, 1970
	DNA fragmentation	Stich et al., 1973;
	DNA repair	San and Stich, 1975
- Urine extracts	Fish tumors	Campbell et al., 1974
HOST-MEDIATED ASSAY		
- S. typhimurium	Mutations	Gabridge and Legator, 1969;
- N. crassa		Legator, 1970;
- S. cerevisiae		Legator and Malling, 1971
- Mouse lymphoma	Mutations	Clive et al., 1973; Fischer, 1973
- Human lymphocytes	Chromosome aberrations	Fischer et al., 1974; Brewen, 1975

3.4.5.1 Cell Transformation Assay--

Cell transformation refers to the conversion of normal cells in culture to malignant cells through the action of a chemical, virus, or other carcinogen. Cell transformation is evidenced by the formation of colonies with the following characteristics:

- Piled-up, criss-cross, spindle-shaped cells
- Hereditary random growth pattern
- Loss of contact inhibition and polar orientation
- Ability to grow indefinitely in vitro
- Relatively resistant to the toxic action of the carcinogen
- Stain heavily with Giemsa
- Cause cancer in animals

Normal cells in culture, on the other hand, form a confluent monolayer of polar-oriented cells of normal morphology which are susceptible to the toxic action of carcinogens and have a limited life span in vitro; they do not cause cancer when injected into animals.

The mechanism of malignant transformation of cells in culture by chemical carcinogens has not been determined as yet. Three theories, however, with a certain amount of evidence in their support, have been advanced (Chen and Heidelberger, 1969):

- Direct transformation of normal cells to cancer cells
- Activation of a latent cancer virus
- Selection of pre-existing cancer cells

The two main physiologic abnormalities manifested by mammalian cells transformed by chemical carcinogens in culture are:

- An increased aerobic glycolysis
- Inhibition of respiration by addition of glucose in presence of pyruvate (Crabtree Effect)

These changes are characteristic of many malignant cell lines and appear to be correlated with the grade of malignancy (Sato et al., 1970).

The species of serum employed in the cultivation of cells in vitro appears to have considerable influence on the development of neoplastic transformation. Sanford et al. (1972) found that C3H mouse embryo cell cultures grown in NCTC-B5 medium containing gelding horse serum regularly underwent malignant transformation between 98-188 days in culture.

Evans et al. (1972), in a follow-up study, found that fetal bovine serum and mare serum delayed neoplastic transformation of the mouse cell line whereas stallion and gelding horse serum hastened the change. Neoplastic transformation was found to be not associated with growth-stimulating capacity of the sera but was believed to be related to the hormones present.

The major disadvantages in the use of cell transformation as a carcinogenicity assay method are (Anon., 1971; Anon., 1973):

- High rate of "spontaneous cell transformation" in certain cell lines, particularly mouse embryo cultures. This does not appear to be a problem, however, with hamster embryo cells.
- The effect of culture medium constituents, such as the species of serum, on transformation.
- Lack of definitive changes in cell morphology which are indicative of transformation in some cell systems, e.g., rat liver. Certain other lines, however, such as hamster embryo, mouse prostate, mouse and rat embryo cultures show characteristic morphological changes when treated with carcinogens.
- Necessity of confirming positive results by animal inoculation.
- A standard activation system is not available for precarcinogens which require metabolic activation to produce malignancy. However, on the basis of results with rat liver cultures obtained by Williams et al. (1973), rodent liver microsomal preparations such as used in the Ames mutagenicity test may be satisfactory.
- Methodology

Among the cell lines in use for screening chemicals for carcinogenicity are: Hamster embryo cells; C3H mouse prostate cells; rat liver cells; rat embryo cells; mouse embryo cells; Syrian hamster chondrocytes; and 3T3 cell line (derived from C3H mouse embryos).

A protocol for in-vitro transformation of BALB/3T3 cells is given in the following pages.

- Carcinogens detected by cell transformation assays

Carcinogenic chemicals which have been detected in vitro by cell transformation assay systems listed above are presented in Table 3.4.9.

- Sensitivity of cell transformation assays

Strong carcinogens induce cell transformation in the assay systems described above at levels as low as 0.01 μg/ml (Table 3.4.10).

3.4.5.2 Unscheduled DNA Synthesis (DNA Repair Synthesis) Assay--

Unscheduled DNA synthesis or DNA repair synthesis bioassays are based upon the fact that most cells are capable of repairing certain types of DNA damage brought about by X-rays, chemicals, etc., by enzymatically excising the damaged portion, resynthesizing the correct sequence of components, and inserting and sealing the new portion of the cellular DNA strand. Nondividing cells must be used so that DNA repair synthesis is not overshadowed by normal s-phase replicative DNA synthesis. DNA repair synthesis can be measured by the incorporation of DNA precursors, such as tritiated thymidine (3H-TdR) into cellular DNA by nondividing cells, by means of autoradiography or scintillation counting.

DNA repair synthesis has not been used as widely as cell transformation

TABLE 3.4.9 TRANSFORMATION OF CELL CULTURES BY CARCINOGENS IN VITRO

Carcinogen	Cell Culture	Reference
3-Methylcholanthrene	Rat embryo	Marquardt and Heidelberger (1972)
	Mouse embryo	Marquardt and Heidelberger (1972)
	Hamster chondrocytes	Katoh (1977
11-Methylcylopenta(a)-phenanthrene	Hamster embryo	Di Paolo et al. (1972)
Dimethylnitrosamine	Hamster embryo	Huberman et al. (1968)
N-Nitrosomethylurea	Rat liver	Williams et al. (1973)
N-2 Fluorenylacetamide	Hamster embryo	Di Paolo et al. (1972)
N-Acetoxy-2-fluorenylacetamide	Hamster embryo	Di Paolo et al. (1972)
N-Hydroxy-N-2-fluorenylacetamide	Hamster embryo	Di Paolo et al. (1972)
4-Nitroquinoline-1-oxide	Hamster embryo	Sato et al. (1970)
	Hamster embryo	Di Paolo et al. (1972)
	Hamster chondrocytes	Katoh (1977)
4-Hydroxyaminoquinoline-N-oxide	Hamster embryo	Di Paolo et al. (1972)
Aflatoxin B_1	Rat liver	Williams et al. (1973)
N-Methyl-N-Nitro-N-Nitrosoguanidine	Hamster embryo	Di Paolo et al. (1972)
Methylazoxymethanol	Hamster embryo	Di Paolo et al. (1972)
Cigarette smoke condensate	Hamster embryo	Rhim and Huebner (1973)
Polycyclic hydrocarbons	Hamster embryo	Di Paolo et al. (1972)
Benzanthracene	Hamster embryo	Benedict et al. (1972)
10-Methylbenzanthracene	Hamster embryo	Berwald and Sachs (1965)
1,2,5,6-Dibenzanthracene	Mouse prostate	Chen and Heidelberger(1969)
	Hamster embryo	Berwald and Sachs (1965)
7,12-Dimethylbenzanthracene	Rat embryo	Rhim and Huebner (1973)
	Rat liver	Williams et al. (1973)
	Mouse embryo	Marquardt and Heidelberger (1972)
9,10-Dimethylbenzanthracene	Mouse prostate	Chen and Heidelberger(1969)
4-Fluoro-10-methyl-1,2-benzanthracene	Mouse prostate	Chen and Heidelberger(1969)
	Hamster embryo	Berwald and Sachs (1965)
	Mouse prostate	Chen and Heidelberger(1969)
3,4-Benzo(a)pyrene	Hamster embryo	Benedict et al (1972)
	Hamster embryo	Huberman et al (1976)
3-Hyroxybenz(a)pyrene	Hamster embryo	Benedict et al. (1972)
	Hamster embryo	Berwald and Sachs (1965)
	Mouse prostate	Chen and Heidelberger(1969)

Continued

TABLE 3.4.9 (Continued)

Carcinogen	Cell Culture	Reference
Cigarette tar	Hamster lung	Inui and Takayama (1971)
City smog	Rat embryo	Freeman et al. (1971)
Negative Chemicals		
Urethane	Hamster embryo	Berwald and Sachs (1963)
	Hamster embryo	Di Paolo et al. (1972)
N-Hydroxyurethane	Hamster embryo	Di Paolo et al. (1972)
Diethylnitrosamine	Hamster embryo	Di Paolo et al. (1972)
1,2,3,4-Dibenzanthracene	Mouse prostate	Chen and Heidelberger (1969)
2-Fluoro-10-methyl-1,2-benzanthracene	Mouse prostate	Chen and Heidelberger (1969)
8-Methylbenz(a)anthracene	Hamster embryo	Berwald and Sachs (1965)
Pyrene	Mouse prostate	Chen and Heidelberger(1969)
	Hamster embryo	Berwald and Sachs (1965)
Chrysene	Hamster embryo	Berwald and Sachs (1965)

TABLE 3.4.10 SENSITIVITY OF CELL TRANSFORMATION ASSAYS

Dose	Carcinogen	Assay System	Treatment Period	Transformation observed	Reference
5 µg/ml	MCA	Hamster chondrocytes	3 days	41-61 days	Katoh (1977)
1 µg/ml	BP	Hamster embryo	3 days		Huberman et al. (1976)
0.05 µg/ml	AB_1	Rat liver	10 weeks	32 days	Williams et al. (1973)
0.01 µg/ml	DMBA	Rat embryo	6 days	40-43 days	Rhim and Huebner (1973)
0.01 µg/ml	BP	Hamster embryo	7 days	7 days	Benedict et al. (1972)
0.03 µg/ml	3-HO-BP	Hamster embryo	7 days	7 days	Benedict et al. (1972)
6.00 µg/ml	BA	Hamster embryo	7 days	7 days	Benedict et al. (1972)
0.015 µg/ml	DMBA	Hamster embryo	7 days	7 days	Benedict et al. (1972)
1.0 µg/ml	MCA	Mouse prostate	6 days	126 days	Chen and Heidelberger (1969)
0.4 µg/ml	MCA	Mouse prostate	6 days	10-14 days	Chen and Heidelberger (1969)

MCA - 3-Methylcholanthrene

BP - Benzo(a)pyrene

AB_1 - Aflatoxin B_1

DMBA - 7,12-Dimethylbenzanthracene

3-HO-BP - 3-Hydroxybenz(a)pyrene

BA - Benzanthracene

for carcinogenicity testing of chemicals but two studies of considerable magnitude have indicated the potential value of this system in detecting chemical carcinogens.

- Human fibroblast cell cultures

Stich et al. (1975) tested 98 different carcinogens, precarcinogens, and noncarcinogens in a DNA repair synthesis assay system consisting of cultured human fibroblasts.

All but two of 29 carcinogens gave positive results in the assay (6.9% false negatives); all 28 noncarcinogens yielded negative results (0% false positives); 11 of 30 precarcinogens were negative and apparently require metabolic activation to induce malignancy.

- Rat Liver Cell Cultures

Williams (1977) used a primary rat liver cell culture system for carcinogenicity testing of chemicals based on the fact that the liver contains all of the enzyme systems for metabolizing all chemical precarcinogens known to require metabolic activation. All compounds were strongly positive with the exception of one weak carcinogen and the 4 noncarcinogenic control chemicals.

The author points out that primary cultures must be used since continuous cell lines lose much of their metabolic activity and would be insensitive to precarcinogens.

EXAMPLE: CELLULAR BIOASSAY
IN-VITRO TRANSFORMATION OF BALB/3T3 CELLS

Purpose of Study

- Carcinogenicity determination

Design of Experiment

- The transformation system used is a quantitative assay that is both rapid and reliable. This method, established by Kakunaga (Int. J. Cancer 12:463-473, 1973), is not only quantitative but scoring for transformed clones is quite clear-cut and reproducible from run to run. It appears to be an ideal screening system for determining the potential of chemicals to induce malignancy.

- An assay consists of a positive control, a vehicle control (negative control), and four dose levels of the test chemical. The length of time required for testing will be 6 to 8 weeks.

Materials

- Assays will be performed using a subclone (obtained from Dr. Takeo Kakunaga) derived from a clone of BALB/3T3. The cells are grown in Eagle's

MEM supplemented with 10% fetal calf serum. The cells are passaged weekly in 60 mm culture dishes.

Conduct of the Experiment

- Seeding: Approximately 10,000 cells are seeded into a 60 mm plastic plate and incubated 24 hours to firmly attach the cells. This plate will be used to assess transformation. Simultaneously with seeding, separate plates will be seeded at 200 cells per plate to obtain toxicity determination. QUALITY CONTROL -- The vehicle for the test chemical is used in the negative control plates.

- Dosing: The positive control and four doses of test chemical are added to the transformation and toxicity plates. Treatment with the test chemicals will consist of exposing the cells in an airtight enclosed chamber to either vapors or gaseous state of the test materials. Various dose levels will be achieved by varying the length of exposure to a fixed level of the vapors or gas. Treatment will be terminated by removing the plates from the chamber and replacing the media with fresh growth media.

- Incubation: Following treatment, the cells will be incubated for 3 to 4 weeks before scoring for transformed foci. The toxicity plates will be scored after only one week. During the incubation periods, growth media will be changed twice weekly.

Observations and Tests

- The transformation plates are aspirated to remove media and washed with buffered saline. The plates are stained with Giemsa, washed, and air dried.

- Transformed clones appear as darkly stained foci on a light background. The counts of the transformation and toxicity plates are then expressed as foci/surviving cells for each dose level. QUALITY CONTROL -- Confirmation of Tumorigenicity of Transformed Clones: Most transformed clones will produce malignant tumors when collected from an unstained transformation plate and injected into syngenic host animals. This confirmation step can be conducted if desired.

Activation

- The BALB/3T3 cells have a limited metabolic capacity but appear to metabolize certain classes of chemicals that have strict requirements for metabolic activation to ultimate carcinogens.

3.4.5.3 Quality Control Aspects--

Quality control procedures for the carcinogenicity testing of chemicals with cell cultures have not been developed to any appreciable extent up to this time. Although precautions and control measures are emphasized by most workers in their reports, no formal system of quality control or quality assurance has been developed.

General quality control measures that should be observed in all work with cell cultures are outlined in Section 3.4.4 of this report. Various specific measures that are especially applicable to carcinogenicity testing with mammalian cells are given in the following pages.

Many chemical carcinogens are light-sensitive and must be stored in the dark. Light should be reduced to a minimum in preparing test solutions and treating cell cultures. Treated cultures, of course, must be incubated in the dark (Katoh, 1977).

Compounds not certified to be pure should be repurified before use. A number of source chemicals and reagents used in organic syntheses are strongly carcinogenic. However, technical grades and formulations of all commercial chemicals should be tested as well as the purified form. Environmental chemicals should also be tested as mixtures since synergistic as well as antagonistic effects may result (Anon., 1973).

Only the cell cultures recommended here should be used and protocols should be followed explicitly in all assays. Primary rat liver cells should be used in all tests involving this system with precarcinogens or unknown chemicals since continuous cell lines lose metabolic activity (Williams, 1977). All assay procedures should be calibrated by the laboratory before testing is started.

Untreated and vehicle control cultures are especially important in cell transformation studies since "spontaneous transformation" to malignancy at a relatively high rate is characteristic of certain cell lines (Anon., 1971; Anon., 1973; Earle, 1943; Berwald and Sachs, 1965). Positive controls in the form of known carcinogens and precarcinogens should also be included in each assay.

Replicate testing and interlaboratory cooperative tests are also advisable in view of the limited data available with most of the cell culture assay systems now being used for carcinogenicity testing of chemicals.

All positive transformation tests must be confirmed by formation of tumors in animals following injection of transformed cells. Correlative data involving transformation manifestations and tumorigenicity in animals should be collected by a central agency or clearinghouse in an effort to define in vitro changes which are sufficient alone as proof of the carcinogenicity of a substance (Anon., 1971).

Cultures used in cell transformation studies must be virus-free since rodents used as source of cells may be parasitized by oncogenic viruses which are capable of transforming cells in culture. Moreover, cultures infected with certain of these viruses are more sensitive to transformation than the corresponding noninfected cultures (Freeman et al., 1971; Rhim and Huebner, 1973; Rapp, 1973).

All lots of serum to be used in cell transformation assays should be tested in untreated cultures as well as in those treated with known carcinogens to ascertain any effect on transformation. In view of results of the studies by Sanford et al. (1972) and Evans et al. (1972), fetal bovine serum is preferable to calf or horse serum.

Glassware used with carcinogens should not be used again for cell transformation carcinogen assays in view of the great sensitivity of these assays and difficulties encountered in removal of the last traces of various carcinogens from containers (Berwald and Sachs, 1965).

3.4.6 Cell Cultures - General Toxicity Testing

Primary cell cultures are especially suitable in general toxicity testing since they retain many of the metabolic and functional characteristics of the original tissues for a number of passages in vitro. The Rabbit Alveolar Macrophage (RAM) Test, Ciliastasis assay, HeLa cell cultures, L-929 mouse fibroblasts, WI-38 human lung fibroblasts, primary rat liver cells, and the Clonal Toxicity Test have been proposed for toxicity testing of air pollutants, pesticides, biomedical materials, and general toxicity of chemicals. Results with several of these systems have been found to correlate well with in vivo assay results (Duke et al., 1977; Donnelly et al., 1974; Litterst et al., 1969; Pelling et al., 1973).

3.4.6.1 Air Pollutants - Test Methods--

- Rabbit Alveolar Macrophage (RAM) Assay

Alveolar macrophages represent a "first line" defense of the mammalian pulmonary system due to their ability to phagocytize and remove particulate material. Consequently, maintenance of viability and phagocytic activity of these cells is essential in protecting the lungs from effects of bacteria and other harmful substances. The Rabbit Alveolar Macrophage (RAM) Assay has been developed as a rapid and convenient in vitro assay for the detection of toxic airborne particulates and associated chemicals. A protocol for this assay is given in the following pages.

Date may be collected on the form illustrated in Figure 3.4.11.

The arc-sine transformation is used in regression analysis (Finney, 1972) since cell viability may be considered to be a binomial response. Viability is plotted against the natural logarithm of the molar concentration.

EXAMPLE: RABBIT ALVEOLAR MACROPHAGE ASSAY (RAM)

Purpose of Study

- Cytotoxicity - Employ rabbit alveolar macrophage to measure quantitatively cellular metabolic impairment and death resulting from exposure in vitro to soluble and particulate toxicants.

Design of Experiment

- Materials. New Zealand white rabbits (including both sexes) weighing 1.5 to 2.0 kg are sacrificed for acquiring alveolar macrophages. Lung lavage in situ is carried out according to the procedure of Coffin et al. (1968) using prewarmed (37°C) sterile 0.85 percent saline. Before conduct of the experiment, make absolutely certain that the following two elements have been properly controlled:

  - Rabbits must be clinically healthy.
  - Determine the cellular composition of the pooled lavage fluid and insure there is routinely 95% alveolar macrophages, 2% to 3% polymorphonuclear leukocytes, and 2% lymphocytes. Discard lavage fluid found to contain blood or mucus.

Conduct of Experiment

QUALITY CONTROL -- Establish regular audits of performance throughout the experiment.

- Cell Culture: The alveolar cells are washed once by centrifugation at 365g for 15 minutes at 25°C and resuspended in prewarmed (37°C) tissue culture medium 199 in Hanks' balanced salt solution. Supplements added to the medium include heat-inactivated fetal bovine serum (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), and kanamycin (100 μg/ml).

  QUALITY CONTROL -- Use supplements in a consistent way. These biologicals will be available from: Gibco, Grand Island, NY.

- Cell Count: The cells are counted by a hemocytometer or automatic cell counter and diluted to approximately 1 x 10^6 cells per ml with supplemented medium.

  QUALITY CONTROL -- Maintain the instruments properly and calibrate them as required.

- Dosing: One ml of the cell suspension is added to each well of 100 x 100 mm 4-place cluster dishes (Falcon Plastics) containing effluent sample, and sufficient medium is added to bring the total volume per well to 2.0 ml. (1) Solid samples: final particle concentrations are 10, 30, 100, 300 and 1000 μg/ml of culture medium, and a control. (2) Liquid samples are added with and without sterile filtration to give a final concentration of 6, 20, 60, 200, and 600 μl/ml, and a control.

  QUALITY CONTROL -- All samples are assayed in a concentration tested in duplicate.

  Randomization.

- Cell Incubation: The cultures are incubated, with rocking, for 20 hours at 37°C in a humidified atmosphere containing 5% CO_2. The pH of the final incubation mixture is recorded before and after incubation.

  QUALITY CONTROL -- No pH adjustments are made for the initial testing. When pH adjustments are made, the sample is tested both with and without adjustments.

- Cell Trypsinization: At the end of the incubation period, the culture medium is poured off and retained separately in a culture tube. Cells are dissociated by using 0.25% trypsin in Gibco solution A. The suspended cells are recombined with the original culture medium and chilled. This trypsinized cell suspension is ready for cell counts, cell viability, total protein, and ATP determination as described below.

- Cell Counts and Viability: (1) Dilute appropriately, usually 4-fold, with cold 0.85% saline to yield a suspension of no more than 2 x 10^5 cells/ml. (2) Add trypan blue, freshly diluted with 0.85% saline to 0.01%, to an equal volume of cell suspension for determination of cell viability. Use a hemocytometer or Cytograf (Biosphysics Systems, Mohapac, NY) to perform simultaneously determinations of cell viability and cell counts.

  QUALITY CONTROL -- All determinations are performed in duplicate. Adequate calibration and proper maintenance of the instrument is essential.

- Protein Determinations: (1) Wash cells twice with 0.85% saline. (2) Lyse cells washed in 1.0% sodium deoxycholate (Schwarz-Mann, Orangeburg, NY) and assay 0.1 ml aliquots of these lysed cells according to the method of Lowry et al. (1951) by using a bovine serum albumin standard (Nutritional Biochemicals Corp., Columbus, Ohio).

  QUALITY CONTROL -- All analyses are made in duplicate. Use standards.

- ATP (Adenosine triphosphate) determinations: Follow DuPont Model 760 Luminescence Biometer procedure. (1) Extract ATP from 0.1 ml aliquot of trypsinized cell suspension containing 0.3 to 0.4 x 10^5 cells with 0.4 ml of dimethylsulfoxide. (2) After 2 minutes at room temperature, buffer the extracted sample with an addition of 5.0 ml cold 0.01 M morpholinopropane sulfonic acid (MOPS) at pH 7.4. (3) Place the tube containing the buffered sample in an ice bath. (4) Inject 10 μl aliquots from (3) into the luminescence meter's reaction cuvette containing 0.7 mM luciferin, 100 units luciferase, and 0.01 M magnesium sulfate in a total volume of 100 μl of 0.01 M MOPS buffer, pH 7.4 at 25°C. (5) Light emitted from the reaction cuvette is measured photometrically in the luminescence meter and proportional to the ATP concentration of the sample.

  QUALITY CONTROL -- All determinations are made in duplicate. Require adequate calibration and proper maintenance for Biometer, as described by manufacturer.

- Phagocytic Activity: (1) Add 1.1 μm polystyrene latex particles (DOW Diagnostics, Indianapolis, Indiana) to alveolar macrophage cultured in Labtek (Miles Laboratories, Inc., Naperville, IL) four-chambered microslides (approximately 25 particles per cell in 1 ml of supplemental medium). Preparation and maintenance conditions are as previously described. (2) One hour after the addition of latex particles, drain slides, air-dry, and expose for 3 minutes to concentrated Wright stain. (3) Expose the slides for an additional 5 to 6 minutes with 1:1 aqueous solution of Wright stain. (4) Air-dry again, and place the slides in xylene for 1 hour to dissolve extracellular particles according to the procedure of Gardner et al. (1974). (5) Air-dry again and mount the slides in permount. (6) Determine the phagocytic activity under oil immersion by scoring a minimum of 200 cells. Each cell which contained at least one particle is considered phagocytically active. Typically, 80% to 90% of control cells ingested one or more latex particles.

QUALITY CONTROL -- Duplicate analysis is made. Needs a quality compound microscope for excellence of work.

Data Collection and Handling

Parameters Measured	Unit	Calculations
Cell counts	Number of cells per milliliter of cell suspension	
Cell viability	Percentage (%)	$\text{Viability index} = \text{Viability (\%)} \times \frac{\text{No. cells experimental}}{\text{No. cells control}}$
Total protein	Percentage (%)	$100\% \times \frac{\text{Experimental total protein}}{\text{Control protein}}$
ATP	Percentage (%)	$100\% \times \frac{\text{Photometric reading of expt.}}{\text{Photometric reading of control}}$
Phagocytic activity	Percentage (%)	$100\% \times \frac{\text{Experimental phagocytic cell counts}}{\text{Control counts}}$

Samples found in the initial screening to significantly affect the parameters being measured are retested for confirmation.

Since cell viability could be considered a binomial response, the arcsine transformation is employed in the regression analysis. Linear relationships of data can be obtained by plotting the transformed viability versus the natural logarithm of the molar concentration. The prediction can be made on the concentration of the test toxicant that yielded a 50% response for any measure parameter (EC50) using a simple regression line. Fifty percent endpoints (EC50) for the various test parameters are obtained through inverse prediction of the simple regression line. All positive samples are retested for confirmation.

References

- The discussion here is principally derived from Section 3.3.2.1 Rabbit Alveolar Macrophage (RAM) Assay in Chapter III, Level 1 Bioassay Techniques of the following report: Duke, K. M., M. E. Davis, and A. J. Dennis. 1977. IERL-RTP Procedures Manual: Level 1, Environmental Assessment Biological Tests for Pilot Studies. EPA-600/7-77-043, April 1977.

Sample No.____________

Date Rec'd____________

Description of Sample____________

____

Date Tested____________

Date Report Out____________

No. Rabbits Used____________

Remarks About Rabbits____________

____

Total No. Cell Recovered________

Seeding Population of Cells_____

DIFFERENTIAL

Macrophages____________

Neutrophils____________

Other____________

Incubation Time____________

EC50 Value____________

Cell Count____________

Viability____________

Viability Index____________

Protein____________

Other____________

TEST RESULTS

Tube No.	Conc. (µg/ml or µl/ml)	pH		Cell No. as % of Control	Viable Cells	Viability Index	ATP*	Protein
		Initial	After Incub.					

* ATP/10^6 cells as % of control

Figure 3.4.11 Data sheet for alveolar macrophage toxicity testing (Duke et al., 1977)

Other references are:

- Coffin, D. L., et al., 1968. Influence of ozone on pulmonary cells. Arch. Environ. Health 16:633-636.

- Gardner, D. E., et al., 1974. Technique for differentiating particles that are cell-associated or ingested by macrophages. Appl. Microbiol. 25:471.

- Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 265-275.

- Mahar, H. 1976. Evaluation of Selected Methods for Chemical and Biological Testing of Industrial Particulate Emissions. EPA-600/2-76-137, or PB-257-912/AS, U.S. Government Printing Office, Washington, D.C.

- Waters, M. D., et al. 1975. Metal toxicity for rabbit alveolar macrophages in vitro. Environ. Res. 9:32-47.

- Waters, M. D., et al., 1974. Screening studies on metallic salts using the rabbit alveolar macrophage in vitro. Environ. Res. 10:342.

- Other Tests

Other cell culture tests for air pollutants are outlined in Table 3.4.11.

TABLE 3.4.11 CELL CULTURE TESTS FOR AIR POLLUTION

Assay	Test Substance	Reference
Rabbit alveolar macrophage (RAM)	Soluble; particulate	Coffin et al., 1968
Human alveolar macrophage	Cigarette smoke	Pratt et al., 1971
Human respiratory epithelial cells (Ciliastasis assay)	Smoke	Ballenger, 1960
Rabbit ciliated epithelial cells	Cigarette smoke	Kensler and Battista, 1963
Rabbit cat tracheal cilia-in vitro/in vivo	Tobacco smoke	Dalhamn, 1970
Rat trachea ciliated epithelia	Chromates	Mass and Lane, 1976

(Continued)

TABLE 3.4.11 (Continued)

Assay	Test Substance	Reference
Freshwater mussel ciliated cells (*Anodonta cataracta*)	Cigarette smoke	Walker and Kiefer, 1966
Hamster tracheal rings	Tobacco smoke	Donnelly et al., 1974

In ciliastasis assay a number of experimental factors have been identified as being critical (Donnelly et al., 1974):

- Species of experimental animal
- Variations in sample preparation
- Test temperatures (pre-chilling of cells is important)
- Control of the ciliostat
- Age of the test sample (loss of volatiles, oxidation, etc.)
- Animal-to-animal and operator-to-operator variations

A randomized complete block design, balancing animal/operator combinations within treatments is recommended.

3.4.6.2 Pesticides--

Pioneer studies on the use of cell cultures for determining pesticide toxicity were made in the middle 1960's. HeLa, KB, human diploid fibroblasts, human Chang liver, and monkey kidney cultures have been employed with a variety of assay techniques and a wide spectrum of pesticides. Comparative studies with HeLa and KB, HeLa and Chang liver, and HeLa and human diploid skin fibroblasts, indicated that sensitivities of the various pairs were very similar for a number of pesticides. Table 3.4.12 outlines tests in use.

TABLE 3.4.12 CELL CULTURE TESTS FOR PESTICIDES

Assay	Test Substances	Reference
HeLa cells	Chlorinated, organophosphorous, or carbamate insecticides	Litterst et al., 1969
	DDA	Johnson and Weiss, 1967
	Various insecticides	Gablicks, 1965
KB cells	DDA	Johnson and Weiss, 1967
Human diploid fibroblasts	Insecticides and metabolites	Litterst and Lichtenstein, 1971
Chang human liver cells	Insecticides	Gablicks and Friedman, 1965

(Continued)

TABLE 3.4.12 (Continued)

Assay	Test Substances	Reference
Monkey kidney cells	Malathion	Desi et al., 1975

3.4.6.3 Biomedical Plastics--

Plastic containers and devices are widely used in the field of human medicine, e.g., containers for transfusion blood, saline, glucose, and other products of injection, in dwelling catheter tubes, heart valves, tracheostomy tubes, transfusion sets, prosthetic devices, etc.

The standard toxicity test method for bioplastics for a number of years has been the rabbit implantation test involving insertion of a small strip of the plastic into the muscle of the animal for 3 to 7 days and examination of the tissue macroscopically and microscopically for evidence of toxicity. Since the method is time-consuming and somewhat expensive, a search has been made for rapid, convenient, and sensitive cell culture tests.

- Leachates from Polymers

In the 1960's, Rosenbluth, Guess, Autian and coworkers developed the L-929 Mouse Fibroblast Cell Culture Assay which correlates well with the in vivo test and is actually more sensitive than the latter (Rosenbluth et al., 1965; Guess et al., 1965).

- Biodegradation Products

Hegyeli and coworkers have developed rapid and quantitative cell culture assays using radiolabelled polymers to predict rates of decay and liberation of toxic substances from plastics intended for use in the body over a very long period of time and which undergo slow biodegradation in vivo (Hegyeli, 1972; Hegyeli et al., 1974). Two procedures used are the Plasma Clot Method and the Organ Culture Method.

Biodegradation rates with a group of cyanoacrylate polymers were as follows:

Polymer	Method	Exposure Period	Degradation Rate
Poly(methyl-2-cyanoacrylate)	Organ culture	24 hr	52.2
	Plasma clot	72 hr	47.4
Poly(ethyl-2-cyanoacrylate)	Organ culture	24 hr	3.1
Poly(propyl-2-cyanoacrylate	Organ culture	24 hr	1.99

(Continued)

Polymer	Method	Exposure Period	Degradation Rate
Poly(butyl-2-cyanoacrylate)	Plasma clot	72 hr	0.66
Poly(isobutyl-2-cyanoacrylate)	Organ culture	24 hr	3.60
Poly(1(+)-lactic acid)	Plasma clot	72 hr	3.50

- Lysosomal Acid-Phosphatase Assay

Grasso et al. (1973) compared the lysosomal acid-phosphatase assay with the agar-overlay cell culture method for toxicity testing of plastics. Primary neonatal rat kidney cell cultures were used in place of L-929 mouse fibroblast cells for both assays. Test plastics employed were samples of polyvinylchloride containing 0, 0.17%, 0.5%, or 1.4% dibutyltin diacetate. Endpoint responses were plaques of dead cells with loss of Neutral Red in the agar-overlay method and increased lysosomal acid-phosphatase activity and loss of Neutral Red in lysosomal assay.

The extent of cell necrosis and other responses was directly proportional to the concentration of dibutyltin diacetate toxicant in both assays. The agar-overlay method was found to be the more sensitive procedure in detecting low concentrations of the toxicant.

- Evaluation of Assays for Toxicity Testing of Medical Plastics

Pelling et al. (1973) evaluated the three major methods for toxicity testing of medical plastics: agar-overlay method, with either (1) L-929 mouse fibroblasts or (2) primary rat kidney cells, and (3) the rabbit implantation test. A variety of plastics used in medical devices were used for the comparative tests.

The L-929 mouse fibroblast assay was the most sensitive method tested. Positive controls produced 2 cm (approx.) plaques of dead (unstained) cells. Negative controls caused no toxicity. The rabbit implantation assay (Sacrospinalis muscle) was more sensitive than implantation in rat subcutaneous tissue. There was good correlation between the L-929 tissue culture assay and the rabbit implantation test although the tissue culture method was considerably more sensitive. The authors suggested that since the L-929 assay is so highly sensitive, positive results should be checked by rabbit implantation tests.

3.4.6.4 General Cellular Toxicity--

- WI-38 Human Lung Fibroblast Assay

The WI-38 strain of human lung fibroblasts is regarded as the best characterized diploid human cell strain available at present for general toxicity testing. All major DNA, RNA, and protein synthesis pathways common to all dividing cells have been found in the WI-38 strain and these cells also possess a number of inducible enzyme systems. WI-38 cell cultures are used by the U.S. EPA Industrial Environmental Research Laboratory, Research Triangle Park, for Level 1 testing of all solid and liquid effluents wherever possible (Duke et al., 1977). A protocol for this assay is given in the following pages. Following the protocol, Figure 3.4.12 gives a form for data collection. Table 3.4.13 outlines other general tests available.

EXAMPLE: HUMAN LUNG FIBROBLAST (WI-38) ASSAY

Human Lung Fibroblast (WI-38) Assay

Purpose of Study

- Cytotoxicity - Employ human lung fibroblasts in culture to measure quantitatively cellular metabolic impairment and death resulting from exposure in vitro to soluble and particulate toxicants.

Design of Experiment

- Normal human diploid WI-38 cells, available from the American Type Culture Collection, Rockville, Maryland, are seeded with 1.75 x 10^5 cells/ml (4.0 ml total volume) in 25 cm^2 Falcon flasks. These cells are grown to confluency in Eagle's basic medium (BME) plus 10% fetal calf serum (FCS). They are then fed with BME plus 0.5% FCS for 5 days.

- Dosages will be determined from preliminary toxicity curves established from treatment with 1.0, 0.10, 0.01 and 0.001% levels of the test compound. Three dose levels of each compound will be selected. A positive and a negative control will also be run.

Conduct of Experiment

QUALITY CONTROL -- Establish regular audits of performance throughout the experiment.

- Culturing: Subcultivate the cultures twice weekly by use of 0.25% trypsin in Gibco solution A (Gibco, Grand Island, NY) with a 1:2 split ratio.
  QUALITY CONTROL -- Cultures should not be employed beyond the 35th subcultivation.
  Use standard culture media.

- Seeding: Cultures or any other subcultures are seeded at 1.75 x 10^5 cells/ml (4.0 ml total volume) in 25 cm^2 Falcon flasks and maintained in Basal Medium Eagle (BME) with Earle's salts plus 10% fetal bovine serum, 2 μmole/ml L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin-B. Cells maintained under these conditions show a period of rapid growth from 24 to 72 hours after subcultivation during which time the experiments are performed.
  QUALITY CONTROL -- Fetal bovine serum must be virus-screened. Routinely antibiotics should be removed from the maintenance to determine the presence of contaminating microorganisms and mycoplasma.

Sample No. ______________

Date Rec'd ______________

Description of Sample ______

____

Date Tested ______________

Date Report Out ___________

Passage of Cells __________

Seeding Population of Cells ___

Incubation Time ___________

EC50 VALUES

Cell Count ______________

Viability ______________

Viability Index ___________

Protein ______________

ATP ______________

Other ______________

TEST RESULTS

Tube No.	Conc. (µg/ml or µl/ml)	pH		Cell No. as % of Control	Viable Cells	Via-bility Index	ATP	Protein
		Initial	After Incub.					

Figure 3.4.12 Data sheet for WI-38 cellular toxicity testing (Duke et al, 1977)

- Dosing: (1) Plant 1.5 to 2.0 x 10^5 cells per flask in 24 cm^2 Falcon flasks.

(2) Add dilutions of the effluent test material 24 hours after the cells have adhered to the flask surface, as described from the RAM (rabbit alveolar macrophage assay).

QUALITY CONTROL -- Each concentration is tested in duplicate.

- Incubation: The culture-effluent mixture is incubated with closed caps for 20 hours at 37°C.

- Trypsinization: At the end of this incubation period, the cells are trypsinized and cell counts, cell viability, protein, and ATP determinations are performed.

- Sample Analysis: Perform cell counts, cell viability, protein, and ATP determinations as described in RAM assay.

QUALITY CONTROL -- All analyses are performed in duplicate. All instruments must be adequately calibrated and in proper maintenance.

Data Collection and Handling

- See description for RAM assay.

Reference

- Duke, K. M., M. E. Davis, and A. J. Dennis. 1977. IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests for Pilot Studies. EPA-600/7-77-043, Office of Research and Development, U.S. Environmental Protection Agency, Washington, D.C.

TABLE 3.4.13 OTHER GENERAL CELLULAR TOXICITY TESTS

Assay	Test Substance	Reference
Clonal toxicity (L929 mouse fibroblast)	Environmental toxicants	Duke et al., 1977
Human KB cells	Misc. chemicals	Smith et al., 1963

3.4.7 Statistical Analysis

An important feature of sound experimentation involves statistical analysis of the data obtained. Since the purpose of a biological assay is to obtain an accurate estimate of the potency of a substance, frequently in relation to a standard, two main types of statistical analysis are involved - estimation of the endpoint and evaluation of results obtained (Finney, 1964). The statistical methods to be applied are determined largely by the general type of experiment, kind of data, results obtained, and so forth.

3.4.7.1 Methods for Calculating a Median Effective Dose--

The biological assays discussed in this section of the report are mainly quantal (yes or no) assays, e.g., death or survival of cells in a treated culture, appearance or absence of a mutant, cessation of ciliary activity, and so forth. The designated endpoint in most quantal bioassays is a median effective dose (ED50, LD50, etc.), i.e., the amount of substance under test which produces a response in fifty percent of the experimental subjects (Finney, 1964). The fifty-percent endpoint is usually chosen because it is more accurate statistically than any other. A number of statistical methods have been developed for estimating fifty-percent endpoints in biological studies. Procedures applicable to quantal assays which have been widely used are:

Reed-Muench Method (Reed and Muench, 1938)
Spearman-Karber Method (Spearman, 1908; Karber, 1931)
Probit Method (Finney, 1964)
Logit Method (Berkson, 1944)
Angle Distribution (Knudsen and Curtis, 1947)
Litchfield-Wilcoxon Method (Litchfield and Wilcoxon, 1949)
Moving Average Method (Thompson, 1947)

In addition to an accurate estimation of the median effective dose, the statistical procedure employed should also permit calculation of 95% confidence limits from the data (Stephan, 1976).

The Reed-Muench method is the least involved procedure for estimation of the ED50, LD50, etc., but, unfortunately, is valid only when the tolerance distribution is symmetrical and requires an unlimited range of doses (Finney, 1964). Moreover, it does not calculate confidence limits or give validity tests. Some leading authorities at least, regard it as statistically inferior to the other methods and state that it should not be used (Finney, 1964; Stephan, 1976).

The original Spearman-Karber method is a rapid and convenient procedure for determination of the median effective dose but cannot always be used in a routine manner since results are biased in some cases (Stephan, 1976). A rather specialized experimental design is also required. The number of subjects per dose should be constant and the doses should be equally spaced (Finney, 1964). For LC50 determinations, the number of doses required is usually large and must cover the complete range from 0% to 100% kill (Stephan, 1976). A modification of this procedure (Armitage and Allen, 1950), however, may be used which does not require a geometric series of doses or equal numbers of test species at each concentration. The method is reported to give approximately the same results as the Probit method if appropriate formulas are used and doses of the test substance cover the complete range from 0% to 100% lethality (Stephan, 1976). Finney (1964) states that the Spearman-Karber method may actually be better than maximum likelihood methods for estimation of the ED50 when the number of subjects per dose is very small.

In quantal assays, the ED50 can also be estimated by converting doses to logarithms and percent effects to probits, logits, or angles followed by application of the curve fitting technique (Litchfield and Wilcoxon, 1949). These three transformations according to Finney (1964) are quite similar over a wide range of responses. In one series of comparative estimations of the ED50 with 12 sets of data, the probit and logit methods agreed very well by the χ^2 test, with the angle transformation being somewhat less satisfactory (Finney, 1964).

The Probit method (Finney, 1964) uses the integrated normal curve and the maximum likelihood curve fitting technique. It can be used regardless of the dosage or number of subjects per dose (Ashton, 1972; Finney, 1964). It also calculates 95% confidence limits and provides validity tests. If the ED50 and standard deviation of the tolerance distribution can be estimated beforehand, Finney (1964) states that the use of the Probit method will give all available information that can be extracted from the records. Stephan (1976) points out that for calculation of the LC50, however, the Probit method can be used only with data which include at least two "partial kills" unless certain of the data are "adjusted". He goes on to state that adjustment of data cannot be justified by any statistical theory. Stephan (1976) also points out that the method is very tedious if a computer or minicomputer is not employed.

The Logit method (Berkson, 1944) uses the logistic curve instead of the integrated normal curve (Ashton, 1972). Ashton (1972) states that although the two curves agree well over the range usually involved in bioassays, the normal curve is the better of the two for this type of study. The logistic curve is best for physicochemical studies (Ashton, 1972). Stephan (1976) points out that for estimation of the LC50, the Logit method like the Probit transformation, is applicable only for data with two or more partial kills, unless the data are "adjusted".

The Angle Transformation (Knudsen and Curtis, 1947) may be used with reasonable confidence that the results will usually be practically the same as if probits or logits had been used provided that extremes of dose, which would cause complications due to the limited range of the tolerance distribution, are not used (Finney, 1964). However, if the number of subjects per dose is either very large or very small, probits or logits are preferable (Finney, 1964). Computations involved in the Angle transformation method are relatively simple, an important feature in routine assays (Finney, 1964).

The Litchfield-Wilcoxon method (1949) can be used, according to Stephan (1976), to estimate the LC50 and its 95% confidence limits from data with one or no partial kills. He points out, however, that it should not be used in cases where the Probit method is not applicable since it was devised as a more convenient method to be used in place of the latter. Furthermore, since it is a semi-graphical method, variation in judgement between individuals may be considerable. Finney (1964) feels that although the Litchfield-Wilcoxon method cannot be recommended without reservation for general use, it may be useful for the professional statistician who will recognize situations in which it will give valid results.

The Moving Average method (Thompson, 1947) is regarded by Stephan (1976) as being usable with more sets of data than any of the other methods, without using adjusted or assumed data. Moreover, it can be performed relatively easily either manually or by computer (Stephan, 1976). Finney (1964), however, calls attention to the fact that a large number of doses are usually required and that the doses should be equally spaced and the number of subjects per dose constant. Also, the tolerance distribution must be symmetrical for a valid estimation of the ED50. Confidence limits cannot be calculated for an LC50 if there are no partial kills, unless "adjusted" data are used (Stephan, 1976).

Finney's recommendations on the choice of a method are as follows:

- If nothing is known about the ED50 beforehand, the following may be used:

  - o Spearman-Karber
  - o Moving Average (with largest possible span)
  - o Probit

  Moving average method is preferred over the Spearman-Karber but is more laborious and probably will give almost the same result as the latter. Probit method will give validity tests which the others do not provide but precision will be little or no greater and it is more laborious. Dose range must be very wide to be certain of bracketing the ED50, and doses should be spaced fairly closely.

- If the experimenter is fairly certain that the ED50 lies between known limits which are not very far apart:

  - o Moving average method (span 3 or greater) is preferable to the Spearman-Karber. In analysis of results, the largest span allowed by data should be used.
  - o Probits may be used if data are unsuitable for the Moving Average method due to an unwise selection of doses. Probits must be used if validity tests are desired.

- If both ED50 and the standard deviation of tolerance distribution can be "guessed" prior to assay, Probits must be used if the experimenter wishes to extract all available information from the records.

The above recommendations assume that statistical advice was followed during planning of the assay (Finney, 1964).

Stephan (1976) makes the following recommendations in his review of methods for calculating an LC50. He points out that they apply also to estimation of LD50, ED50, and EC50 in quantal assays:

- Moving Average method and log concentration is method of choice with one or more partial kills. This method may be used also with no partial kills but confidence limits cannot be calculated under these conditions.
- Probit, Litchfield-Wilcoxon, and Logit methods should be used only

with two or more partial kills.

- Spearman-Karber method should be employed only if both 0% and 100% kill are included in the data.

3.4.7.2 Methods for Assessing Significance of Data--

Procedures for evaluating significance of results obtained are discussed in Section 2.2.3.

3.4.8 References

Akin, E. W., and W. Jakubowski. 1976. Viruses in finished water: The Occoquan experience. Proc. AWWA Water Qual. Technol. Conf., San Diego, Calif., Dec. 5-8.

Ames, B. N., and C. Y. Yanofsky. 1971. The detection of chemical mutagens with enteric bacteria. In: Chemical Mutagens: Principles and Methods for their Detection, Vol. 1. A. Hollaender (ed.). Plenum Press, New York, N.Y. pp. 267-282.

Ames, B. N., W. E. Durston, E. Yamasaki, and F. D. Lee. 1973a. Carcinogens as mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc. Nat. Acad. Sci. U.S.A. 70(8): 2281-2285.

Ames, B. N., F. D. Lee, and W. E. Durston. 1973b. An improved bacterial test system for the detection and classification of mutagens as carcinogens. Proc. Nat. Acad. Sci. U.S.A. 70: 782-786.

Ames, B. N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with Salmonella/mammalian microsome mutagenicity test. Mutat. Res. 31: 347-364.

Anon. 1971. Panel on Carcinogenesis Report on Cancer Testing in the Safety Evaluation of Food Additives and Pesticides, FDA Advisory Committee on Protocols For Safety Evaluation. Toxicol. Appl. Pharmacol. 20: 419-438.

Anon. 1972. Evaluation and testing of drugs for mutagenicity. World Health Organ. Chron. 26(2): 69-72.

Anon. 1973. The Testing of Chemicals for Carcinogenicity, Mutagenicity, and Teratogenicity. Ministry of Health and Welfare of Canada, Ottawa, Canada. pp. 1-39.

Anon. 1975. The Testing of Chemicals for Carcinogenicity, Mutagenicity, and Teratogenicity. Ministry of Health and Welfare of Canada, Ottawa, Canada. pp. 69-134.

Armitage, P., and I. Allen. 1950. Methods of estimating the LD50 in quantal response data. J. Hyg. 48:298-322.

Ashton, W. D. 1972. The logit transformation, with special reference to its uses in bioassay. Charles Griffin and Co., Ltd., London, England.

Auerbach, C., J. M. Robson, and J. D. Carr. 1947. The chemical production of mutations. Science 105: 243-247.

Bailey, W. R., and E. G. Scott. 1970 Diagnostic Microbiology. C. V. Mosby Co., St. Louis, Mo.

Ballenger, J. J. 1960. Experimental effect of cigarette smoke on human respiratory cilia. New Engl. J. Med. 263: 832-835.

Barile, M. F. 1973. Mycoplasma contamination of cell cultures: Incidence, source, prevention, and problems of elimination. In: Tissue Culture: Methods and Application. P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.). Academic Press, New York, N.Y. pp. 729-735.

Barry, A. L., and K. L. Feeny. 1967. Quality control in bacteriology through media monitoring. Amer. J. Med. Technol. 33(5): 387-393.

Bartlett, R. C., W. R. Irving, and C. Rutz. 1968. Quality Control in Clinical Microbiology. American Society of Clinical Pathology, Commission on Continuing Education, Chicago, Ill.

Bartlett, R. C. 1973. Functional quality control. In: Quality Control in Microbiology. J. E. Prier, J. T. Bartola, and H. Friedman (eds.). University Park Press, Baltimore, Md. pp. 145-184.

Benedict, W. F., J. E. Gielen, and D. W. Nebert. 1972. Polycyclic hydrocarbon-produced toxicity, transformation, and chromosomal abberation as a function of aryl hydrocarbon hydroxylase activity in cell cultures. Int. J. Cancer 9: 435-451.

Berg, G., D. R. Dahling, and D. Berman. 1971. Recovery of small quantities of virus from clean waters on cellulose nitrate membrane filters. Appl. Microbiol. 22: 608-614.

Berkson, J. 1944. Application of the logistic function to bioassay. J. Amer. Statist. Ass. 39: 357-365.

Berwald, Y., and L. Sachs. 1963. In vitro cell transformation with chemical carcinogens. Nature 200(4912): 1182-1184.

Berwald, Y., and L. Sachs. 1965. In vitro transformation of normal cells to tumor cells by carcinogenic hydrocarbons. J. Nat. Cancer Inst. 35(4): 641-661.

Bicking, C. A. 1976. The sampling of biological materials. In: Proceedings of a Seminar on Sampling of Bulk Materials, European Organization for Quality Control. STAQUAREL 1976, Prague, Czechoslovakia.

Blazevic, D. J., C. T. Hall, and M. E. Wilson. 1976. Practical Quality Control Proceedings for the Clinical Microbiology Laboratory. American Society for Microbiology, Washington, D.C.

Bodily, H. L., E. L. Updyke, and J. O. Mason (eds.). 1970. Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections, 5th Edition. American Public Health Association, New York, N.Y.

Boone, C. W. 1973. A surveillance procedure applied to sera. In: Tissue Culture: Methods and Application. P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.). Academic Press, New York, N.Y. pp. 677-682.

Brusick, D., and H. Andrews. 1974. Comparison of the genetic activity of dimethylnitrosamine, ethylmethanesulfonate, 2-acetylaminofluorene and ICR-170 in Saccharomyces cerevisiae strains, D3, D4, and D5 using in vitro assays with and without metabolic activation. Mutat. Res. 26: 491-500.

Brusick, D., R. J. Weir, and H. E. Giss. 1976. Screening program for the identification of potential chemical mutagens and carcinogens. Litton Bionetics, Inc., Kensington, Md.

Buchanan, R. E., and N. E. Gibbons (eds.). 1974. Bergey's Manual of Determinative Bacteriology. Williams and Wilkins Co., Baltimore, Md.

Capizzi, R. L., W. J. Smith, R. Field, and B. Papirmeister. 1973. A host-mediated assay for chemical mutagens using the L5178Y/asn murine leukemia. Mutat. Res. 21: 6.

CDC. 1969. Recommended Specifications for Microbiological Reagents, 3rd Edition. Center for Disease Control, Department of Health, Education and Welfare, Atlanta, Ga.

Chen, T. T., and C. Heidelberger. 1969. Quantitative studies on the malignant transformation of mouse prostate cells by carcinogenic hydrocarbons in vitro. Int. J. Cancer 4: 166-178.

Cherry, W. B., M. Goldman, T. R. Carski, and M. D. Moody. 1960. Fluorescent Antibody Techniques in the Diagnosis of Communicable Disease. Center for Disease Control, Department of Health, Education and Welfare, Atlanta, Ga.

Chu, E. H. Y. 1971. Induction and analysis of gene mutations in mammalian cells in culture. In: Chemical Mutagens: Principles and Methods for Their Detection. Vol. 2. A. Hollaender (ed.). Plenum Press, New York, N.Y. pp. 411-444.

Chu, E. H. Y., and H. V. Malling. 1968. Mammalian cell genetics. II. Chemical induction of specific locus mutations in Chinese hamster cells in vitro. Proc. Nat. Acad. Sci. U.S.A. 61: 1306-1312.

Clarkson, J. M., and H. J. Evans. 1972. Unscheduled DNA synthesis in human leucocytes after exposure to UV light, gamma rays, and chemical mutagens. Mutat. Res. 14: 413-430.

Clayson, D. B. 1973. Historical evaluation of short-term tests for carcinogenicity. In: Carcinogenesis Testing of Chemicals. Proceedings of a Conference on Carcinogenesis Testing in the Development of New Drugs, May 23-25, 1973, Washington, D.C. L. Goldberg (ed.). CRC Press, Cleveland, Ohio. pp. 79-89.

Clive, D., W. G. Flamm, and J. B. Patterson. 1973. Specific locus mutational assay systems for mouse lymphoma cells. In: Chemical Mutagens: Principles and Methods for Their Detection. Vol. 3. A. Hollaender (ed.). Plenum Press, New York, N.Y. pp. 79-104.

Clive, D., and J. F. S. Spector. 1975. Laboratory procedures for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutat. Res. 31: 17-29.

Cliver, D. O. 1967. Enterovirus detection by membrane chromatography. In: Transmission of Viruses by the Water Route. G. Berg (ed.). Interscience Publishing Co., New York, N.Y.

Coffin, D. L., D. E. Gardner, R. S. Holzman, and F. S. Wolach. 1968. Influence of ozone on pulmonary cells. Arch. Environ. Health 16: 633-636.

Coriell, L. L. 1973a. Laboratory applications of laminar flow. In: Quality Control in Microbiology. J. E. Prier, J. T. Bartola, and H. Friedman (eds.). University Park Press, Baltimore, Md., pp. 41-46.

Coriell, L. L. 1973b. Glassware preparation, sterilization, and use of laminar flow systems. In: Tissue Culture: Methods and Application. P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.). Academic Press, New York, N.Y. pp. 671-673.

Craun, G. F., L. J. McCabe, and J. M. Hughes. 1976. Waterborne disease. Outbreaks in the U.S. - 1971-1974. J. Amer. Water Works Ass. 68(8): 4106-4110.

Dalhamn, T. 1970. _In vivo_ and _in vitro_ ciliatoxic effects of tobacco smoke. Arch. Environ. Health 21: 633-634.

Dalhamn, T., and R. Rylander. 1969. Experimental requirements for the toxicological evaluation of tobacco smoke in the respiratory system. Scand. J. Resp. Dis. 50: 273-280.

Davies, R. R. 1971. Air sampling for fungi, pollens, and bacteria. In: Methods in Microbiology. Vol. 4. J. R. Norris and D. W. Ribbons (eds.). Academic Press, New York, N.Y. pp. 367-404.

de Serres, F. J. 1974. Mutagenesis assessment. Report of Discussion Group No. 12. In: Carcinogenesis Testing of Chemicals. Proceedings of a Conference on Carcinogenesis Testing in the Development of New Drugs, May 23-25, Washington, D.C. L. Goldberg (ed.). CRC Press, Cleveland, Ohio. pp. 101-107.

de Serres, F. J., and H. V. Malling. 1971. Measurement of recessive lethal damage over the entire genome and at two specific loci in the ade-3 region of a two-component heterokaryon of _Neurospora crassa_. In: Chemical Mutagens: Principles and Methods for Their Detection. Vol 2. A. Hollaender (ed.). Plenum Press, New York, N.Y. pp. 311-342.

Desi, I., G. Dura, L. Gonczi, Z. Kneffel, A. Strohmayer, and Z. Szabo. 1975. Toxicity of malathion to mammals, aquatic organisms, and tissue culture cells. Arch. Environ. Contam. Toxicol. 3: 410-425.

Di Paolo, J. A., R. L. Nelson, and P. J. Donovan. 1972. In vitro transformation of Syrian hamster embryo cells by diverse chemical carcinogens. Nature 235(5336): 278-280.

Donnelly, G. M., H. E. McKean, C. S. Heird, and J. Green. 1974. Ciliostasis as a bioassay. Source of variations and their control. Arch. Environ. Health 28: 350-355.

Duke, K. M., M. E. Davis, and A. J. Dennis. 1977. IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests for Pilot Studies. U.S. Environmental Protection Agency, EPA-600/7-77-043, Office of Research and Development, Washington, D.C.

Earle, W. R. 1943. Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in living cells. Cancer Inst. 4: 165-212.

Ellis, R. J. 1976. Manual of Quality Control Procedures for Microbiological Laboratories, 2nd Edition. Center for Disease Control, Department of Health, Education and Welfare, Atlanta, Ga.

Epstein, S. S., M. Small, J. Kaplan, N. Mantel, and S. H. Hunter. 1963. Photodynamic bioassay of benzo(a)pyrene with Paramecium caudatum. J. Nat. Cancer Inst. 31: 163-168.

Evans, V. J., F. M. Price, K. K. Sanford, H. A. Kerr, and S. L. Handleman. 1972. Comparative effects of mare, stallion, gelding horse, and fetal bovine sera on neoplastic transformation in vitro. J. Nat. Cancer Inst. 49: 505-512.

Fahrig, R. 1975. Development of host-mediated mutagenicity tests - yeast systems. II. Recovery of yeast cells out of testes, liver, lung, and peritoneum of rats. Mutat. Res. 31: 381-394.

Finney, D. J. 1964. Statistical Method in Biological Assay. 2nd Edition, Charles Griffin and Co., Ltd., London, England

Finney, D. J. 1972. Probit Analysis. 3rd Edition. Cambridge University Press, London, England. pp. 76-80.

Flamm, W. G. 1974. A tier system approach to mutagen testing. Mutat. Res. 26: 329-333.

Freeman, A. E., P. J. Price, R. J. Ryan, R. G. Gordon, R. V. Gilden, G. J. Kelloff, and R. J. Huebner. 1971. Transformation of rat and hamster embryo cells by extracts of city smog. Proc. Nat. Acad. Sci. U.S.A. 68: 445-449.

Gablicks, J. 1965. Response of cell cultures to insecticides. II. Chronic toxicity and induced resistance. Proc. Soc. Exp. Biol. Med. 120: 168-171.

Gablicks, J., and L. Friedman. 1965. Response of cell cultures to insecticides. I. Acute toxicity to human cells. Proc. Soc. Exp. Biol. Med. 120: 163-171.

Gabridge, M. G., and M. S. Legator. 1969. A host-mediated microbial assay for the detection of mutagenic compounds. Proc. Soc. Exp. Biol. Med. 130: 831-834.

Geldreich, E. E. 1975. Handbook for Evaluating Water Bacteriological Laboratories. 2nd Edition. U.S. Environmental Protection Agency, EPA-670/9-75-006, Cincinnati, Ohio.

Giever, P. M. 1976. Particulate matter sampling and sizing. In: Air Pollution 3rd Edition, Vol. 3. A. C. Stern (ed.). Academic Press, New York, N.Y. pp. 3-50.

Glasser, L., G. S. Bosley, and J. R. Boring, III. 1971. A systematic program of quality control in clinical microbiology. Amer. J. Clin. Pathol. 56(3): 379-383.

Goddard, K. R. 1976. Measurements and calibration of air quality, particulate matter, and microbial levels. Workshop C, First Annual Conference on Quality Programs and Government Regulations, Arlington, Va. Apr. 12-13.

Grasso, P., J. Gaydon, and R. J. Hendy. 1973. The safety testing of medical plastics. II. An assessment of lysosomal changes as an index of toxicity in cell cultures. Fd. Cosmet. Toxicol. 11: 255-263.

Gray, J. P., and J. R. Kennedy. 1974. Ultrastructure and physiological effects of non-tobacco cigarettes on _Tetrahymena_. Arch. Environ. Health 28: 283-291.

Gray, R. D., and G. H. Lowe. 1976. The preparation of simulated water samples for the purpose of bacteriologic quality control. J. Hyg. 76(1): 49-56.

Green, S., E. Zeiger, K. A. Palmer, J. A. Springer, and M. S. Legator. 1976. Protocols for the dominant lethal test, host-mediated assay, and _in vivo_ cytogenetic test used in the Food and Drug Administration's review of substances in the GRAS (Generally Recognized as Safe) list. J. Toxicol. Environ. Health 1: 921-928.

Greene, A. E., and J. Charney. 1973. Serological identification of cells in culture. B. Poikilotherm cell identity by cytotoxic antibody test. In: Tissue Culture: Methods and Application. P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.). Academic Press, New York, N.Y. pp. 750-753.

Guess, W. L., S. A. Rosenbluth, B. Schmidt, and J. Autian. 1965. Agar dispersion method for toxicity screening of plastics on cultured cell monolayers. J. Pharm. Sci. 54: 1545-1547.

Halstead, E. G., R. A. Quevedo, and W. H. Gingerich. 1971. A quality control program for the bacteriology laboratory. Amer. J. Med. Technol. 37(1): 15-20.

Hayflick, L. 1973. Screening tissue cultures for *Mycoplasma* infection. In: Tissue Culture: Methods and Application. P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.). Academic Press, New York, N.Y. pp. 722-728.

Hebert, A. G., B. Pittman, R. M. McKinney, and W. B. Cherry. 1972. The Preparation and Physical Chemical Characteristics of Fluorescent Antibody Reagents. Center for Disease Control, Department of Health, Education and Welfare, Atlanta, Ga.

Heden, C-G., and T. Illeni (eds.). 1974. Automation in Microbiology and Immunology. John Wiley and Sons, New York, N.Y.

Hegyeli, A. F. 1972. The use of organ cultures to assess biodegradation of polymers. In Vitro 7:267.

Hegyeli, A. F., J. C. Eaton, R. M. Rice, S. J. Gourley, J. G. Dillon, and R. K. Kularni. 1974. *In vitro* biodegradation and biocompatibility studies. In Vitro 9: 374.

Hill, W. F., Jr., W. Jakubowski, E. W. Akin, and N. A. Clarke. 1976. Detection of viruses in water: Sensitivity of the tentative standard method for drinking water. Appl. Environ. Microbiol. 31(2): 254-261.

Hsu, T. C. 1973. Karyology of cells in culture. A. Preparation and analysis of karyotypes and idograms. In: Tissue Culture: Methods and Application. P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.). Academic Press, New York, N.Y. pp. 764-767.

Huberman, E., S. Salzberg, and L. Sachs. 1968. The *in vitro* induction of an increase in cell multiplication and cellular life span by the water-soluble carcinogen dimethylnitrosamine. Proc. Nat. Acad. Sci. U.S.A. 59(1): 77-82.

Huberman, E., R. Mager, and L. Sachs. 1976. Mutagenesis and transformation of normal cells by chemical carcinogens. Nature 264(5584): 360-361.

Huisingh, K. 1976. Personal communication. Cellular Biology Branch, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Triangle Park, N.C.

Inui, N., and S. Takayama. 1971. Effects of cigarette tar upon tissue culture cells. Brit. J. Cancer 25: 574-583.

Jacob, M. I. 1958. The growth and morphology of *Tetrahymena* as influenced by aminoazobenzene dyes and certain metabolic inhibitors. Dissert. Abstr. 18: 368.

Jacobson, A. R., and S. C. Morris. 1976. The primary air pollutants - Viable particulates, their occurrence, sources, and effects. In: Air Pollution, 3rd Edition, Vol. 1. A. C. Stern (ed.). Academic Press, New York, N.Y. pp. 169-196.

Jakubowski, W., J. C. Hoff, N. C. Anthony, and W. F. Hill, Jr. 1974. Epoxy-fiberglass adsorbent for concentrating viruses from large volumes of potable water. Appl. Microbiol. 28: 501.

Jawetz, E., J. L. Melnick, and E. A. Adelberg. 1972. Review of Medical Microbiology, 10th Edition. Lange Medical Publications, Los Altos, Calif.

Johnson, W. J., and S. A. Weiss. 1967. Cytotoxicity of dichlorodiphenylacetic acid (DDA) upon cultured KB and HeLa cells and its reversal by mevalonic acid. Proc. Soc. Exp. Biol. Med. 124: 1005-1008.

Kao, F-T., and T. T. Puck. 1968. Genetics of somatic mammalian cells. VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. Nat. Acad. Sci. U.S.A. 60: 1275-1281.

Karber, G. 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch. Exp. Pathol. Pharm. 162: 480-487.

Katoh, Y. 1977. Transformation and neoplastic development of hamster chondrocytes after exposure to 4-nitroquinoline-1-oxide and 3-methylcholanthrene in tissue culture. J. Nat. Cancer Inst. 59: 155-163.

Kavanagh, F. W. 1974. Automated systems for analytical microbiology. IV. Accuracy measurements. J. Pharm. Sci. 63(9): 1463-1467.

Kennedy, J. R., and A. M. Elliott. 1970. Cigarette smoke: The effect of residue on mitochondrial structure. Science 168: 1097-1098.

Kensler, C. J., and S. P. Battista. 1963. Components of cigarette smoke with ciliary-depressant activity. New Engl. J. Med. 269(22): 1161-1166.

Knudsen, L. F., and J. M. Curtis. 1947. The use of the angular transformation in biological assays. J. Amer. Statist. Ass. 42:282-296.

Kuzel, N. R., and F. W. Kavanagh. 1971. Automated systems for analytical microbiology. I. Theory and design considerations. J. Pharm. Sci. 60(5): 764-767.

LaMotte, L. C., Jr. 1973. Role of CDC in external proficiency testing. In: Quality Control in Microbiology. J. E. Prier, J. T. Bartola, and H. Friedman (eds.). University Park Press, Baltimore, Md. pp. 137-144.

Legator, M. S. 1972. The need to detect chemically-induced mutations in experimental animals. In: Mutagenic Effects of Environmental Contaminants. H. E. Sutton and M. I. Harris (eds.). Academic Press, New York, N.Y. pp. 67-79.

Legator, M. S. 1976. Host-mediated assay. In: First Annual Course in Principles and Practices of Genetic Toxicology. University of Texas Medical Branch at Galveston. Galveston, Texas, September 19-24, 1976.

Legator, M. S., and H. V. Malling. 1971. The host-mediated assay, a practical procedure for evaluating potential mutagenic agents in mammals. In: Chemical Mutagens: Principles and Methods for Their Detection. Vol. 2. A. Hollaender (ed.). Plenum Press, New York, N.Y. pp. 569-589.

Lennette, E. H., and N. J. Schmidt (eds.). 1969. Diagnostic Procedures for Viral and Rickettsial Infections. 4th Edition. American Public Health Association, New York, N.Y.

Lennette, E. H., E. H. Spaulding, and J. P. Truant. 1974. Manual of Clinical Microbiology. 2nd Edition, American Society of Microbiology, Washington, D.C.

Lieberman, M. W., S. Sell, and E. Farber. 1971. Deoxyribonucleoside incorporation and the rule of hydroxyurea in a model lymphocyte system for studying DNA repair in carcinogenesis. Cancer Res. 31: 1297-1312.

Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96(2); 99-113.

Litterst, C. L., E. P. Lichtenstein, and K. Kajiwara. 1969. Effects of insecticides on growth of HeLa cells. J. Agr. Food Chem. 17: 1199-1203.

Litterst, C L., and E. P. Lichtenstein. 1971. Effects and interactions of environmental chemicals on human cells in tissue culture. Arch. Environ. Health 22: 454-459.

Lott, J. A. 1973. Laboratory personnel, the most important aspect of quality control. Med. Instrum. (Balt.) 7(1): 66.

Maramorosch, K., and H. Koprowski (eds.). 1967. Methods in Virology. Academic Press, New York, N.Y.

Marquardt, H., and C. Heidelberger. 1972. Influence of "feeder cells" and inducers and inhibitors of microsomal mixed-function oxidases on hydrocarbon-induced malignant transformation of cells derived from C_3H mouse prostate. Cancer Res. 32: 721-725.

Mass, M. J., and B. P. Lane. 1976. Effect of chromates on ciliated cells of rat tracheal epithelium. Arch. Environ. Health 31: 96-100.

McCann, J., E. Choi, E. Yamasaki, and B. N. Ames. 1975. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci. U.S.A. 72: 5135-5139.

Metcalf, T. J. 1961. Use of membrane filters to facilitate the recovery of viruses from aqueous suspensions. Appl. Microbiol. 9: 376.

Morton, H. E. 1973. Maintenance and use of stock cultures in microbiological quality control. In: Quality Control in Microbiology. J. E. Prier, J. T. Bartola, and H. Friedman (eds.). University Park Press, Baltimore, Md. pp. 55-63.

Ong, T-M., D. F. Callen, S. L. Huang, R. P. Batzinger, and E. Bueding. 1977. Mutation induction by the anti-schistosomal drug F30066 in various test systems. Mutat. Res. 48: 37-42.

Painter, R. B., and J. E. Cleaver. 1969. Repair replication, unscheduled DNA synthesis, and the repair of mammalian DNA. Radiat. Res. 37: 451-466.

Pelling, D., M. Sharratt, and J. Hardy. 1973. The safety testing of medical plastics. I. An assessment of methods. Fd. Cosmet. Toxicol. 11: 69-83.

PHS. 1968. Public Health Service Regulations, Part 74, Title 42, Clinical Laboratories Improvement Act of 1967. Fed. Regist. 33(253): 20043-20049.

Power, D. A. 1973. Quality control of commercially prepared bacteriological media. In: Quality Control in Microbiology. J. E. Prier, J. T. Bartola, and H. Friedman (eds.). University Park Press, Baltimore, Md. pp. 47-53.

Pratt, S. A., M. H. Smith, A. J. Ladman, and T. N. Finley. 1971. The ultrastructure of alveolar macrophages from human cigarette smokers and nonsmokers. Lab. Invest. 24(5): 331-338.

Prier, J. E. 1973. The role of the state public health laboratory in external quality control. In: Quality Control in Microbiology. J. E. Prier, J. T. Bartola, and H. Friedman (eds.). University Park Press, Baltimore, Md. pp. 123-135.

Prier, J. E., J. T. Bartola, and H. Friedman (eds.). 1973. Quality Control in Microbiology. University Park Press, Baltimore, Md.

Pumper, R. W. 1973. Purification and standardization of water for tissue culture. In: Tissue Culture: Methods and Application. P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.). Academic Press, New York, N.Y. pp. 674-677.

Rand, M. C., A. E. Greenberg, and M. J. Taras (eds.). 1975. Standard Methods for the Examination of Water and Wastewater. 14th Edition. American Public Health Association, American Waterworks Association, and Water Pollution Control Federation, Washington, D.C. pp. 871-880; 904-908; 954-956; 968-974.

Rapp, E. 1973. Malignant transformation of cells in culture. In: Tissue Culture: Methods and Applications. P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.). Academic Press, New York, N.Y. pp. 653-658.

Ray, V. A., H. E. Holden, D. S. Salsburg, J. H. Ellis, Jr., L. J. Lust, and M. H. Voyer. 1973. Comparative studies of induced mutations with host-mediated, dominant lethal, and cytogenetic assays. Mutat. Res. 21: 12.

Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty-percent endpoints. Amer. J. Hyg. 27: 493-497.

Rhim, J. S., and R. J. Huebner. 1973. Transformation of rat embryo cells in vitro by chemical carcinogens. Cancer Res. 33: 695-700.

Rippere, R. A., and B. Arret. 1972. Automated turbidimetric microbiologic assay readout systems. J. Pharm. Sci. 61(3); 449-454.

Rosenbluth, S. A., G. R. Weddington, W. L. Guess, and J. Autian. 1965. Tissue culture method for screening toxicity of plastic materials to be used in medical practice. J. Pharm. Sci. 54(1): 156-159.

Russell, R. L. 1974. Quality control in microbiology. In: Manual of Clinical Microbiology, 2nd Edition. E. H. Lennette, E. H. Spaulding, and J. P. Truant. American Society for Microbiology, Washington, D.C. pp. 862-878.

Russell, R. L., Y. S. Yoshimori, T. F. Rhodes, J. W. Reynolds, and E. R. Jennings. 1969. A quality control program for clinical microbiology. Amer. J. Clin. Pathol. 52(4): 489-494.

Sanford, K. K., J. L. Jackson, R. Parshad, and R. R. Gant. 1972. Evidence for an inhibitory influence of fetal bovine serum on "spontaneous" neoplastic transformation in vitro. J. Nat. Cancer Inst. 49: 513-518.

Sato, K., T. Kuroki, and H. Sato. 1970. Respiration and glycolysis of cells transformed with 4-nitroquinoline-1-oxide and its derivatives. Proc. Soc. Exp. Biol. Med. 134: 281-283.

Sato, K., R. S. Slesinski, and J. W. Littlefield. 1972. Chemical mutagens at the phosphoribosyltransferase locus in cultured human lymphoblasts. Proc. Nat. Acad. Sci. U.S.A. 69(5): 1244-1248.

Shapiro, N.I., A. E. Khalizer, E. V. Luss, M. I. Marshak, O. N. Petrova, and N. B. Varshaver. 1972. Mutagenesis in cultured mammalian cells. I. Spontaneous gene mutations in human and Chinese hamster cells. Mutat. Res. 15: 203-214.

Slater, E. E., M. D. Anderson, and H. S. Rosenkranz. 1971. Rapid detection of mutagens and carcinogens. Cancer Res. 31: 970-973.

Smith, C. G., J. E. Grady, and J. I. Northam. 1963. Relationship between cytotoxicity in vitro and whole animal toxicity. Cancer Chemother. Rep. 30: 9-12.

Spearman, C. 1908. The method of "right and wrong cases" "constant stimuli" without Gauss' formulae. Brit. J. Psychol. 2:227-242.

Stephan, C. E. 1976. Methods for calculating an LC50. In: Aquatic Toxicology and Hazard Evaluation. F. L. Mayer and J. L. Hamelink (eds.). Proceedings of a Symposium Sponsored by the ASTM Committee E-35 on Pesticides. Memphis, Tenn., October 25-26, 1976.

Stich, H. F., and R. H. C. San. 1970. DNA repair and chromatid anomalies in mammalian cells exposed to 4-nitroquinoline-1-oxide. Mutat. Res. 10: 389-404.

Stich, H. F., R. H. C. San, and Y. Kawazoe. 1971. DNA repair synthesis in mammalian cells exposed to a series of oncogenic and non-oncogenic derivatives of 4-nitroquinoline-1-oxide. Nature 229(5284): 416-419.

Stich, H. F., P. Law, L. W. Lo, D. J. Koropatnick, and R. H. C. San. 1975. The search for relevant short-term bioassays for chemical carcinogens: The tribulations of a modern sisyphus. Can. J. Genet. Cytol. 17(4): 471-492.

Stoltz, D. R., L. A. Poirier, C. C. Irving, H. F. Stich, J. H. Weisburger, and H. C. Grice. 1974. Evaluation of short-term tests for carcinogenicity. Toxicol. Appl. Pharmacol. 29: 157-180.

Stulberg, C. S., and W. F. Simpson. 1973. Serologic identification of cells in culture. A. Animal cells by fluorescent-labelled antibody. In: Tissue Culture: Methods and Applications. P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.). Academic Press, New York, N.Y. pp. 744-749.

Suggs, M. T. 1973. Product class standards (specifications) and evaluation of microbiological in vitro diagnostic reagents. In: Quality Control in Microbiology. J. E. Prier, J. T. Bartola, and H. Friedman (eds.). University Park Press, Baltimore, Md. pp. 87-104.

Thompson, W. R. 1947. Use of moving averages and interpolation to estimate median-effective dose. I. Fundamental formulas, estimation of error, and relation to other methods. Bacteriol. Rev. 11:115-145.

U.S. EPA. 1977. Draft of mutagenicity testing requirements. FIFRA, Section 3, Guidelines on Hazard Evaluation of Humans and Domestic Animals. U.S. Environmental Protection Agency, Office of Pesticide Programs, Washington, D.C.

U.S. Pharmacopeia XIX. 1976. Mack Publishing Co., Easton, Pa.

Vera, H. D. 1971. Quality control in diagnostic microbiology. Health Lab. Sci. 8(3): 176-189.

Walker, T. R., and J. E. Kiefer. 1966. Ciliastatic components in the gas phase of cigarette smoke. Science 153: 1248-1250.

Wallis, C., and J. L. Melnick. 1967. Concentration of viruses from sewage by adsorption on millipore membranes. Bull. World Health Organ. 36: 219.

Wallis, C., M. Henderson, and J. L. Melnick. 1972. Enterovirus concentration on cellulose membranes. Appl. Microbiol. 23: 476.

Wang, H. 1963. Differential responses of Paramecium aurelia to cigarette components. Nature 197(4871): 946-948.

Webber, B. B., and F. J. de Serres. 1965. Induction kinetics and genetic analysis of X-ray-induced mutations in the ad-3 region of Neurospora crassa. Proc. Nat. Acad. Sci. U.S.A. 53: 430.

Weiss, W. 1965. The toxicity of tobacco smoke solutions for Paramecium. The influence of various forms of filtration. Arch. Environ. Health 10: 904-909.

Weiss, W. 1968. Cigarette smoke gas phase and Paramecium survival. Arch. Environ. Health 17: 62-64.

Weiss, W., and W. A. Weiss. 1964. Effect of tobacco smoke solutions on Paramecium. Arch. Environ. Health 9: 500-504.

Weiss, W., and W. A. Weiss. 1967. The gas phase toxicity of cigarette smoke for Paramecium. Arch. Environ. Health 14: 682-686.

WHO Scientific Group. 1971. Evaluation and testing of drugs for mutagenicity: Principles and problems. World Health Organ., Tech. Rep. Ser. 482: 5-18.

Williams, G. M. 1977. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cultures. Cancer Res. 37: 1845-1851.

Williams, G. M., J. M. Elliott, and J. H. Weisburger. 1973. Carcinoma after malignant conversion in vitro of epithelial-like cells from rat liver following exposure to chemical carcinogens. Cancer Res. 33: 606-612.

Wilson, M. E. 1973. Microbiological proficiency: What basis for confidence? In: Quality Control in Microbiology. J. E. Prier, J. T. Bartola, and H. Friedman (eds.). University Park Press, Baltimore, Md. pp. 119-122.

Woodard, G. 1976. Draft Survey and Evaluation of in vitro Toxicity Test Methods. U.S. Environmental Protection Agency, EPA 560/5-75-007.

Wynder, E. L., and D. Hoffman. 1964. Experimental tobacco carcinogenesis. Advan. Cancer Res. 8: 249-453.

Zeiger, E., and D. Brusick. 1971. The host-mediated assay - a protocol for Salmonella and Saccharomyces. Environ. Mutagen Soc. Newsletter 5: 32-34.

Zimmermann, F. K. 1973. Detection of genetically-active chemicals using various yeast systems. In: Chemical Mutagens: Principles and Methods for Their Detection. Vol. 3. A. Hollaender (ed.). Plenum Press, New York, N.Y. pp. 209-239.

Zimmermann, F. K. 1975. Procedures used in the induction of mitotic recombination and mutation in the yeast *Saccharomyces cerevisiae*. Mutat. Res. 31: 71-86.

3.5 MAMMALIAN BIOASSAY

3.5.1 Experimental Design Aspects

The principal goal of any mammalian bioassay experimental design is to ensure, as far as possible, that no agents, physical factors, or biologic organisms, except those under test or used as treatments, contribute to or influence the observed result. This ideal is generally very difficult to achieve. In practice in mammalian bioassays, the best that can be done is to ensure that all factors, influences, or conditions, except one, the treatment agent, act equally on two populations, the exposed, test or treatment group and the control group (that group not exposed to the treatment agent). It is important to emphasize that no valid conclusions can be drawn from an uncontrolled experiment.

The control group ought to be handled wherever possible in the exact same manner as the test group. The very act of administering an unknown or toxic agent may influence the course of a bioassay and the observed results, even though the agent may be inert or only weakly toxic. A simulated treatment using a known inert substance, termed a placebo control, is often used to avoid this difficulty, since the method of administration or dosing of an animal may produce the dominant adverse effect if the stress is overly great on an animal. Placebo controls in a pilot study should be included to elucidate such potential problems (Goldstein, 1964).

Good experimental design with mammals generally requires that the controls be run concurrently with the treatment groups within a simultaneous experimental situation. "Before-after" comparisons generally make for weak logic and inferences. For example, if a previous group of animals had a high acute mortality against a specific agent while a second group of control animals observed later showed little or no mortality, it may be argued that the test group could have died mainly from secondary causes (e.g., infections, contaminated water or feed, or intracage fighting).

In some unique instances it may be possible to use each animal as its own control. Such designs which incorporate each animal as internal controls have been used in skin and eye irritancy or allergenicity tests where more than one area or organ is available to the investigator, one of which receives placebo treatment. There is always a formal requirement that the test agent in pilot studies show only localized adverse effects. Any agent which readily migrates from the area of treatment via systemic transfer within a specified time period cannot be validly bioassayed using this internal control method. Such is the case for chemicals which distribute within an animal's body following rapid percutaneous absorption (e.g., solvents such as acetone or ethanol), but not the case for more reactive or more polar agents such as formaldehyde or diethylthalamide which have been demonstrated to produce positive skin irritations in the Draize (internal control) dermatitis assay (Steinberg et al., 1975).

In some instances where the toxic responses, particularly in a chronic toxicity or carcinogenicity test, may vary with time or be brought into question due to changing physiology with age, it may be necessary to assay a positive control group along with the placebo controls and test groups. Positive controls may also be necessary in the case of potentially weak carcinogens where a clear-cut interpretation of the histopathologic observations requires concurrent analysis of results of a known carcinogen for comparison in a given species and/or strain of animal (Weisburger, 1975).

In all the above examples, the objective of using control populations has been to isolate the effects, whether they be acute toxicity, mutagenicity, or carcinogenicity, resulting from the interaction of only the test agent on the organism. It is thus necessary that there be equivalence, as nearly complete as possible, between the housing, feeding, watering, dosing, examination schedule, and posting in both the control and the test animals. Even then, the resulting observations are strictly valid only for the conditions of the original bioassay. The strength of any correlations and inferences with respect to human health evaluations and exposures must, therefore, rest on the relative merits of the experimental designs and techniques employed in the bioassay and not just on the actual statistical numbers or observations made.

Randomization in the selection of animals for use as test and control groups is another means of achieving the necessary concurrent testing equivalence between these groups. Randomization prevents most subconscious bias which has been demonstrated to play a surprisingly large role in the assignment of mammals in a given trial. For instance, if the investigator chooses only those animals that are the slowest and easiest to catch for a test group, while the remaining friskier animals are used as controls, one may point to the possibility that the two populations are not equivalent. In fact, the test group may be fatter or metabolically impaired in some unknown way allowing them to be more easily caught. Goldstein (1964) succinctly observed that "no characteristic of a subject whatsoever shall play any part in his assignment to a group" in a properly run trial, since sound statistical inferences rely on the assumption of random distribution or assignment of subjects or individual organisms.

The blind design is a preferred approach because bias in the conduct of the trial or bioassay and in the evaluation of results is eliminated or minimized. In the blind design the personnel who administer, observe, score and/or evaluate the results are "blind" as to which individual animals belong to a control set and which belong to test sets, usually by means of coding of dosage materials. The strength of the blind design lies in the high degree of objectivity that it affords the personnel and investigators directly involved in the trial progress and evaluation.

The basic design in bioassay is a single factor design (a single toxic substance is under test at a given time) with replication (treatment applied to more than one animal in a group). Usually the treatment (factor) is applied at more than one level and these levels are fixed (not chosen at random).

All the considerations mentioned above apply also to experiments with more than one factor and where some additional considerations apply.

The general definition for balanced design is one involving two or more factors in which comparisons are made between treatment groups in such a way that all factors except the treatment of interest affect the treatment groups equally. A special case of the balanced design called a cross-over design is useful when a bioassay is to be repeated or when several different exposures are to be assessed over an extended period of time. In the cross-over design, the test group and control group in the first trial are exchanged in the second trial.

Obviously trials involving acute toxicities which lead to permanent injury or death of the test subjects cannot be performed in this manner.

When more than a single control set and test set is involved, then more elaborate designs such as the Latin square, randomized block, or factorial designs may be advantageous. The placement of the animal cages within an animal room may be moderated via one of these designs so as to eliminate the possibility of skewed effects due to adverse lighting, noise, vibration, temperature, etc., at one end of a room. Factorial designs have been discussed in Sect. 2.2 which also given references to sources of more elaborate designs.

When a sequence of exposures, treatments, or observations is needed within a trial (not necessarily a time sequence) a nested or hierarchical design is recommended whereby a tier of trials is performed in a specific sequence. This method might be used to assess the performance of several instruments, technicians and/or laboratories in terms of the accuracy and precision through replication of procedures and assays. The enhanced reliability of the data obtained may be crucial for discussions involving safe limits of human exposure or in determining "no-effect" levels to wildlife.

The test sample size is also of primary importance in an experimental design since the statistics based on this sample will generally be used as estimators of the corresponding parameters and data in larger populations. For a specific level of significance (e.g., $P<0.05$), the Type I, or alpha error (i.e., the probability of assigning a significantly positive cause-and-effect relationship, when in fact the reverse is true) is specified at the beginning of a trial by the investigator. By contrast, the Type II, or beta error (i.e., the probability of assigning a non-significant cause-and-effect correlation, when in fact this correlation is significant) is a complex function of several factors, one of which is the alpha error chosen. If one can tolerate a larger alpha error (say $P<0.05$ instead of $P<0.01$), then the beta error is made concomitantly smaller. Secondly, as the sample size is increased, the distributions between the control sample and the test sample will become narrower due to the "central limit" theorem and less overlap between these distributions will result. As stated by Sokal and Rohlf (1969), the "central limit" theorem predicts that as the sample size increases, the means of samples drawn from a population of any distribution will approach a normal or Gaussian distribution. Moreover, if the sample values already fit a Gaussian distribution, then increasing the sample size has the effect of narrowing that distribution (i.e., reducing the variance) about the central tendency (i.e., the mean of the sample values).

One final approach to reducing the beta error is to choose that measurement parameter which is most readily influenced by the treatment, drug, toxicant, etc. Thus, for example, if the earliest and most sensitive measures for inhalation intoxication are cardiac and respiratory rates, while recording of electroencephalographic activity recordings are slower to change, then the former, more sensitive responses will yield the least beta error when cause-and-effect correlations are drawn.

3.5.2 Conditions of Test

3.5.2.1 Compounding the Test Material--

The general principles involved in the preparation of various dosage forms are discussed here with reference to the in vivo delivery of test materials. Besides route of administration, a number of factors influence the extent of test agent bioavailability, that is, the degree to which the test agent in a specific dosage form is available for absorption, distribution, biotransformation, and physiologic action. Among the factors that affect bioavailability are:

- Stability and chemical purity
- Particle size and/or crystalline form
- Diluents and/or excipients including fillers, binders, disintegrating agents, lubricants, coatings, solvents, and suspending agents
- Method of manufacture and/or compounding which may cause chemical and/or physical degradation, introduces metal or packaging contaminants (active or inactive)

The U.S. Pharmacopeia XIX explicitly states that "the maintenance of a demonstrably high degree of bioavailability requires particular attention to all aspects of production and quality control that may affect the nature of the finished dosage forms" (U.S. Pharmacopeia, 1975).

The setting of specification standards for products requires a statement of the expected shelf-life for each product or preparation, which in turn requires knowledge of stability (i.e., the time lapse from initial preparation during which a dosage form continues to fulfill specifications for identity, strength, quality and purity). It has been recommended (U.S. Pharmacopeia, 1975) that analytical methodology should be cited capable of differentiating between intact preparations and the degradation products thereof. In addition, stability is prolonged by storage at optimum environmental conditions (i.e., generally low temperature, humidity, air and light). Four types of stability criteria should be checked in order to certify that a specific shelf-life is accurate:

- Chemical - each active ingredient retains its chemical integrity and labeled potency, within the specified limits
- Physical - the original physical properties, including appearance, uniformity, dissolution and suspendability, are retained
- Microbiological - sterility or resistance to microbial growth is retained according to the specified requirement; antimicrobial agents that are present retain effectiveness within

the specified limits

- Toxicological - no significant increase in toxicity occurs

Though not all these criteria are fully applicable to the testing of all toxicants (e.g., it might in fact be the objective of a study to determine how and what toxicants are chemically formed when storage conditions for a pesticide are far from optimum), proper toxicologic investigations should require delineation of these stability criteria, especially before and at the end of a chronic feeding experiment when the possibility is very high that a toxicant is chemically, physically, or microbiologically altered with time.

3.5.2.2 Vehicles--

Capsules and tablets are the two most common forms of oral administration of test compounds to humans, while other mammals are orally dosed chiefly by water or feed supplemented with test compounds or less often by gavage with liquid formulations (Weisburger, 1976; Goodman and Gilman, 1975). In most animal tests, the oral route (supplementation of water or feed or dosing by gavage) is generally the most practical and reliable approach in bioassaying for acute, subacute or chronic toxicity, or carcinogenicity. Gavage or per os (po) administration offers the advantage (or disadvantage) of quantitative dosage delivery using one entire dose at a time. Absorption of test compounds from dietary water or feed, on the other hand, has the advantage (or disadvantage) of protracting the animals' exposure, leading to a greater possibility of biotransformation. An important consideration here is that the animals may be offended by the taste of the test compound and reduce their intake of food and/or water, making for poor comparisons with control animals. Moreover, checks should be made of the uniformity of distribution of the test chemical in feed by quantitative chemical analyses before exposing the animals in the proposed bioassay.

Dosing via inhalation can be quite complex requiring exposure of nasal, respiratory and/or oral tracts in a uniform (between animals) and/or consistent (within an animal) manner before a valid assessment of local and/or systemic effects can be made. Nebulizers are suitable for the administration of inhalation solution only if these instruments can be certified to give droplets of requisite and uniform size distribution so that the dosing mist is assured of reaching the bronchioles. Devices that release a metered dose via aerosolization with a liquified propellant of "inert" gas should first be tested without test substance to determine side effects, if any, of the propellant. Upon addition of the test agent, the device should again be tested to ascertain whether the specified particle size distribution and amounts released by the aerosol are achieved.

3.5.2.3 Routes of Administration--

Administration of test substances to test mammals should incorporate those routes that most closely approximate the routes whereby humans become exposed (Weisburger, 1976; U.S. FDA, 1959). If the chosen route of absorption is to be via the gastrointestinal tract (enteral), then three oral routes of administration to animals are possible:

- Gavage (gastric intubation)
- Mixing into solid diet
- Mixing into drinking water

The gavage method has the advantages of (a) ease of quantification, (b) use of minimal test agent, (c) provision of fresh test preparations, and (d) ease of storage, but the disadvantages of (a) a large amount of animal handling, (b) high hazard of lung and/or esophageal damage within the test animals with subsequent increases in non-toxicant-induced mortality, (c) a requirement for small and sometimes concentrated volumes of toxicant, (d) use of a solvent, and (e) need for close weight-matching of test animals. Solid diet mixing affords: (a) a greater total intake of test agents, (b) close simulation of the mode of human exposure, (c) lessening of the hazards to the animals' lungs and esophagus, (d) reduction of the requirement for solvents, and (e) avoidance of the requirement for close weight-matching of animals.

When the animal feed is to be supplemented with the test agent, a number of potential problems, however, must be taken into consideration such as (a) lack of homogeneity of the mix, (b) decomposition and/or interaction of the toxicant with the feed during storage, (c) adverse palatability of the mix, (d) varying quantity of ingestion between individual animals yielding only average estimates, and (e) contamination of the feed with synergistic or antagonistic substances that may go unnoticed or be uncontrollable. Compared with dietary exposure, similar advantages and disadvantages exist for mixing toxicants into the animals' drinking water except that inhomogeneity is less of a problem, while decomposition due to hydrolysis may be a major objection to dosage via drinking water.

Some common parenteral routes of administering liquid test substances, which obviate problems associated with the gastrointestinal tract (e.g., slow absorption, gastric decomposition, or precipitation), are compiled below in Table 3.5.1 along with the major uses, limitations and precautions involved in each technique. The instantaneous or prompt absorption and complete systemic distribution via these parenteral routes ensure complete and uniform dosing of the test animals, but require great care in their handling and in avoidance of overdosing. The above routes all have in common that the toxicant or test substance will rapidly (parenteral routes) or eventually (enteral routes) reach a nearly uniform systemic distribution in the test organisms' bodies prior to being concentrated in various tissues and organs, causing reversible or irreversible physiologic changes, and usually resulting in a termination of biologic action by means of tissue-organ-specific biotransformation and elimination.

Localized pharmacologic or toxic actions, however, may be produced by application of test substances to various specific locations on the skin and eye, by inhalation into the lungs and nasal passages, and by other mucosal, especially sublingual (beneath the tongue), applications. Cutaneous or dermal entry of test agents generally requires the use of a solvent which has been demonstrated to be nonirritating. Elicitation of a dermal reaction by the combined toxicant-solvent formulation is thus proof positive of absorptive cutaneous toxicity, whereas the lack of a dermal reaction may indicate poor or nonabsorption across the epidermal stratum corneum rather than lack of

TABLE 3.5.1 CHARACTERISTICS OF COMMON ROUTES OF TOXICANT ADMINISTRATION (Goodman and Gilman, 1975)

Route	Pattern	Special Utility	Limitations and Precautions
Intravenous (iv)	Absorption instantaneous with potential for immediate physiologic effects.	Permits titration of dose and use in an emergency. Suitable for large volumes and irritating substrates and drugs if diluted.	Must introduce substances slowly, as a rule, and watch for increased risk of adverse effects. Not suitable for oily solutions, particulate materials, or insoluble substances.
Subcutaneous (sc)	Prompt absorption from aqueous solution, but permitting slow and sustained distribution from repository preparations.	Suitable for some insoluble suspensions and for implantation of solid pellets.	Not suitable for large volumes. May cause slough from irritating substances.
Intramuscular (im)	Prompt absorption from aqueous solution, but permitting slow and sustained distribution from repository preparations.	Suitable for moderate volumes, oily vehicles, and some irritating substances.	May interfere with interpretation of some diagnostic tests (e.g., creatine phosphokinase).
Intraperitoneal (ip)	Same as intramuscular, but can be quicker.	Same as intramuscular.	May cause infections and/or adhesions. Not used on man.
Oral ingestion (per os, po)	Variable absorption depending on gastric pH, gastric emptying rate, dissolution rate of solids, powders, crystals, coatings or capsules, etc.	Most convenient, safe and economical dosing method.	Absorption potentially erratic and incomplete for agents that are poorly soluble and absorbed slowly. Agents which are degraded or destroyed by gastric acids and enzymes are precluded from dosing in this manner.

cutaneous toxicity. A primary irritant has been defined by the FDA as a substance producing an injury on first contact. The resultant injury will depend on:

- the nature of the irritant-solvent combination
- the concentration of the irritant
- the total duration of the first exposure

Primary dermal irritation may be measured via the patch-test technique on intact or abraded skin clipped free of hair. The irritation process, an incipient inflammation, may vary from barely perceptible hyperemia, to edema and vesiculation, to erythema, and finally to intense suppurative processes. Numerous methods of primary irritancy (PI) quantification have been reported, but the Draize approach is widely accepted as the method of choice (Steinberg et al., 1975).

Ocular PI is also of great concern. Because of the vital role they play in vision, the FDA recommends that injuries to the cornea and iris be weighted more heavily than injury to bulbar and palpebral conjuctivae (U.S. FDA, 1959). The cornea, having 40% of a score, is rated on the basis of the density of induced opacity and the amount of area involved while the iris, also weighted 40%, is scored on the intensity or degree of inflammation exhibited. The conjunctivae, including the cornea, iris, palpebral and remaining bulbar mucosae, are scored for a total of 20% and are rated on the basis of the degree of chemosis, redness, and discharge.

Other mucosae, i.e., oral, genito-urinal and rectal, are subject to wider variations than ocular muscosa in toxic responsiveness due to changes in tissue pH, contact with food and microbes, secretions, excretions, and absorptive capacity, on an hourly, daily, or monthly basis making intertest comparison difficult. These mucosae, however, generally exhibit faster absorption due to the lack of a cornified barrier (i.e., the stratum corneum) than absorption through intact skin, with the result that the necessity of compounding of the test agent with a solvent may be avoided.

In contrast to the single localized exposure PI bioassays described above, sensitization or contact allergy studies on skin, eye or mucosae are studies which result in:

- tissue reactions that are remote from the original site of test application
- enhancement of responsiveness with each subsequently applied equal dosage
- potentiation of the cell-mediated, as well as the antibody-mediated, immune systems

Reactions such as erythema and/or edema are generally not observed upon first contact with the toxicant but become increasingly manifest when further exposures occur. The number of prior exposures to application of agents required to produce these allergic responses may vary greatly depending on sensitizing potential of the agents, the species susceptibility and the number of exposures to dosages at a wide range of concentrations. Sensitization is scored on the basis of the number of positively reactive animals and on the degree of their reactivity. It should be noted, however, that many classes of substances exist (e.g., poison ivy allergens) which induce contact sensitivity in humans but for which natural animal models are rare or non-existent.

Systemic dermal toxicity, chiefly measured in terms of acute or subacute lethality following cutaneous exposure, is generally more difficult to assay than toxicity studies resulting from ingestion or inhalation due to the fact that the animal must be restrained from licking the skin or inhaling toxic vapor arising therefrom. In acute single exposure studies, the agent may be held in skin contact by means of a rubber sleeve with a reservoir containing test agent for periods varying up to 24 hours. Multiple dosage 20-day and 90-day subacute dermal toxicity studies are most difficult to execute properly due to the requirements for preventing exposure via inhalation or ingestion during the entire course of these assays. Progressive deterioration of the skin may thus be investigated together with protracted damage to other tissues and organs as a result of the eventual systemic distribution of cutaneously absorbed toxicants.

Gases or aerosol preparations are best tested by acute or subacute inhalation toxicity tests, in addition to skin mucous membrane assays. In the case of aerosols, an important quality control criterion would be the size distribution of the aerosolized particles. Particles in the range of less than 3 µm readily reach and deposit in the alveolar sacs of mammalian lungs, whereas particles of 3 to 10 µm arrive at the lung parenchyma with great difficulty. Particles larger than 10 µm are effectively prevented from reaching the parenchyma and alveoli and would therefore provide false tests of lung intoxication. A closely confined test space or exposure chamber is a necessity for each control and test animal.

In acute inhalation testing the objective is to assay the test animals using single or multiple dosages of gas or aerosol within a short period (e.g., less than 8 hours). The U.S. FDA (1959) recommends that the animals be immobilized within the test chambers with a suitable covering placed over their eyes. Immobilization allows the investigator to direct the release of aerosol or gas around the head and upper trunk of the animal, while avoiding the release of spray directly into the animal's respiratory passage with the possibility of causing a bolus toxic effect. A minimum of 4 test animals is required by the FDA for determining acute effects of aerosol preparations.

Their recommended procedure, following immobilization of each animal (one per chamber), is to dose for 30 seconds of continuous spray release concomitant with a 15-minute continuous ensuing exposure before the 30-second dosage is repeated. At 30-minute intervals the spray release is repeated until a minimum of 10 successive exposures has been completed. In order to control extraneous routes of exposure, it is recommended that each animal's fur and body be cleaned of extraneous test substances before placement in a standard maintenance cage. Over the ensuing 4-day period, observations of symptomatology, food intake, body weight changes and hematology are recommended at a minimum, to be followed by sacrifice of the animals and histopathology of their tissues and organs.

Subacute inhalation assays are required once or several times in daily repetitive testing over a considerable length of the animals' life. U.S. FDA (1959) mandated that this be a period of 90 days for subacute toxicity assessments. As above, the same exposure chamber and animal cleanup procedure is used, this time with a minimum of five animals. At least two daily 30-second continuous spray exposures several hours apart are required. The parameters of symptomatology, food intake, body weight changes and hematologic morphology are measured daily. At the conclusion of the trial, histopathologic examination of tissues and organs of the sacrificed animals is performed.

3.5.3 Good Animal Care Laboratory Practices (GLP's)

A complete set of good animal care laboratory practices for use in carcinogen bioassay based on the DHEW Guide (ILAR, 1974a) will be found in Appendix B. The following sections comment on the main aspects of the practices useful for all mammalian bioassay.

3.5.3.1 Sources of Animals--

According to the U.S. Department of Health, Education and Welfare, it is recommended that only commercial suppliers that are accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) should serve as sources of laboratory-bred animals. These suppliers must meet AAALAC's criteria based on published standards for good quality, health, housing, hygiene, overall care, feeding, watering, and care by competent veterinarians, breeders and animal care personnel before accreditation is granted. See ILAR (1974a) and associated references (1960 to 1977).

3.5.3.2 Physical Facilities of Quarantine Area--

The quarantine area should be located in rooms physically separated from existing testing areas. Separate rooms should be provided for each species.

Except for relaxed caging requirements prior to distribution, physical conditions during quarantine shall be of the same quality as that provided animals under test.

If an epizootic disease or parasitic infection is found among the animals upon arrival, or at any time during quarantine, the entire shipment should be discarded and the room disinfected prior to the receipt of additional animals.

3.5.3.3 Examination Upon Receipt--

Animals shall be received, in their unopened shipping containers, in the designated quarantine area.

Discard substandard animals on receipt for size, health or other reasons.

Examine all animals for general health. Sacrifice a random sample of 1/20 of the animals and examine for parasites. Palpate all animals and discard any with an abnormality.

3.5.3.4 Caging Before Distribution for Test--

A shipment may be caged together during quarantine, acute toxicity test and repeated dose study according to the weight-space requirements in Table 3.5.2.

TABLE 3.5.2 SPACE RECOMMENDATIONS FOR LABORATORY ANIMALS (ILAR, 1974a)

Species	Weight	Type of Housing	Floor Area/Animal (Square)	Height[1]
Mouse	Up to 10 g	Cage	39 cm (6 in)	12.7 cm (5 in)
	10 – 15 g	"	52 cm (8 in)	12.7 cm (5 in)
	16 – 25 g	"	77 cm (12 in)	12.7 cm (5 in)
	Over 25 g	"	97 cm (15 in)	12.7 cm (5 in)
Rat	Up to 100 g	Cage	110 cm (17 in)	17.8 cm (7 in)
	100 – 200 g	"	148 cm (23 in)	17.8 cm (7 in)
	201 – 300 g	"	187 cm (29 in)	17.8 cm (7 in)
	Over 300 g	"	258 cm (40 in)	17.8 cm (7 in)
Hamster	Up to 60 g	Cage	64.5 cm (10.0 in)	15.2 cm (6 in)
	60 – 80 g	"	83.9 cm (13.0 in)	15.2 cm (6 in)
	81 – 100 g	"	103.2 cm (16.0 in)	15.2 cm (6 in)
	Over 100 g	"	122.6 cm (19.0 in)	15.2 cm (6 in)
Guinea Pig	Up to 250 g	Cage	277 cm (43 in)	17.8 cm (7 in)
	250 – 350 g	"	374 cm (58 in)	17.8 cm (7 in)
	Over 350 g	"	652 cm (101 in)	17.8 cm (7 in)
Rabbit	Up to 2 kg	Cage	.14 m (1.5 ft)	35.6 cm (14 in)
	2 – 4 kg	"	.28 m (3.0 ft)	35.6 cm (14 in)
	Over 4 kg	"	.37 m (4.0 ft)	35.6 cm (14 in)
Cat	Up to 4 kg	Cage	.28 m (3.0 ft)	61.0 cm (24 in)
	Over 4 kg	"	.37 m (4.0 ft)	61.0 cm (24 in)
Dog[2]	Up to 15 kg	Pen or Run	.74 m (8.0 ft)	—
	15 – 30 kg	"	1.12 m (12.0 ft)	—
	Over 30 kg	"	2.23 m (24.0 ft)	—
	Up to 15 kg	Cage	.74 m (8.0 ft)	81.3 cm (32 in)
	15 – 30 kg	"	1.12 m (12.0 ft)	91.4 cm (36 in)
	Over 30 kg	"	REFER TO FOOTNOTE NO. 2	
Primates[3,4]				
Group 1	Up to 1 kg	Cage	.15 m (1.6 ft)	50.8 cm (20 in)
Group 2	Up to 3 kg	"	.28 m (3.0 ft)	76.2 cm (30 in)
Group 3	Up to 15 kg	"	.40 m (4.3 ft)	76.2 cm (30 in)
Group 4	Over 15 kg	"	.74 m (8.0 ft)	91.4 cm (36 in)
Group 5	Over 25 kg	"	2.33 m (25.0 ft)	213.4 cm (84 in)
Pigeon[5]	—	Cage	742 cm (115 in)	—
Chicken[5]	Up to ½ kg	Cage	232.3 cm (36 in)	—
	½ – 2 kg	"	464.5 cm (72 in)	—
	2 – 4 kg	"	1090.4 cm (169 in)	—
	Over 4 kg	"	1651.7 cm (256 in)	—
Sheep and Goat	Up to 25 kg	Pen	0.93 m (10 ft)	—
	25 to 50 kg	"	1.40 m (15 ft)	—
	Over 50 kg	"	1.86 m (20 ft)	
Hog	Up to 50 kg	Pen	.56 m (6 ft)	—
	50 – 100 kg	"	1.12 m (12 ft)	—
	Over 100 kg	"	2.79 m (30 ft)	—
Cattle	Up to 350 kg	Stanchion	1.5 m (16 ft)	—
	350 – 450 kg	"	1.7 m (19 ft)	—
	450 – 550 kg	"	2.0 m (21 ft)	—
	550 – 650 kg	"	2.2 m (24 ft)	—
	Over 650 kg	"	2.5 m (27 ft)	—
	Up to 75 kg	Pen	2.2 m (24 ft)	—
	75 – 200 kg	"	4.7 m (50 ft)	—
	200 – 500 kg	"	9.3 m (100 ft)	—
	500 – 600 kg	"	11.2 m (120 ft)	—
	600 – 700 kg	"	13.0 m (140 ft)	—
	Over 700 kg	"	14.0 m (150 ft)	—
Horse	—	Tie Stall	4.1 m (44 ft)	—
	—	Pen	13.4 m (144 ft)	—

TABLE 3.5.2 (continued)

[1] Height means from the resting floor to the cage top.

[2] These recommendations may require modifications according to the body conformations of particular breeds. As a further general guide, the height of a dog cage should be equal to the height of the dog over the shoulders (at the withers), plus at least six inches, and the width and depth of the cage should be equal to the length of the dog from the tip of the nose to the base of the tail, plus at least six inches.

[3] The primates are grouped according to approximate size with examples of species that may be included in each group:

Group 1—Marmosets, tupaias, and infants of various species.
Group 2—Cebus and similar species.
Group 3—Macaques and large African species.
Group 4—Baboons, monkeys larger than 15 kg. and adult members of brachiating species such as gibbons, spider monkeys and woolly monkeys.
Group 5—Great Apes.

[4] Where primates are housed in groups in pens, only compatible animals should be kept. Minimum height of pens should be six feet. Resting perches, nesting boxes and escape barriers necessary for the well being of the particular animals should also be provided.

[5] Sufficient headroom must be provided so birds can stand erect without crouching.

3.5.3.5 Quarantine Period--

o Animals should be quarantined for a minimum of 7 days.

3.5.3.6 Reexamination of Animals--

At the end of the quarantine period, the animals should be reexamined for health (and palpated) and any additional substandard ones discarded.

If a sufficient number of healthy animals to satisfy test protocol requirements is on hand after reexamination, they may be distributed for testing. If the number is insufficient, a new supply of animals may need to be obtained and the quarantine and examination repeated.

3.5.3.7 Disposal of Animals Dead on Receipt or During Quarantine--

All procedures involved in the disposal of dead animals shall be in conformance with Federal, State and local laws and regulations pertaining to pollution control and protection of the environment.

Waste cans for use in removal of dead animals should be equipped with leakproof disposable liners and tight-fitting lids.

3.5.3.8 Quality Controls at Quarantine--

Shipments containing dead, moribund, or unsatisfactory animals must be reported immediately to the program management and in writing to the animal supply house concerned, with a copy to the program management.

Results of examination for parasites in individual animals in the sample sacrificed, including all negative findings, should be recorded in a bound laboratory notebook by the clinician performing the examination and witnessed by the laboratory supervisor. It shall be the responsibility of the laboratory supervisor to verify that a complete record has been made for each shipment within 8 days of receipt of the shipment.

3.5.3.9 General Health Requirements--

Hinkle (1977) has stressed that "a good laboratory animal medicine program should provide effective preventive medicine" since there is little reason for using diseased animals in any type of bioassay. Thus, the following quality control checks should be made:

Observation - All animals should be observed regularly by properly qualified personnel for signs of diseases. Animal care should be under direction of veterinarians with specialized training and experience in laboratory animal medicine, especially those certified by the American College of Laboratory Animal Medicine. Sick or moribund animals or animals found dead should be removed from the colony, and a proportion should be examined by laboratory procedures (including pathology) to determine the cause of morbidity or death.

Routine Monitoring Methods - At regularly scheduled intervals, water bottles and feces should be cultured in order to determine whether the predominant organisms present are similar or identical to those previously established and that pathogens are not present. At regularly scheduled intervals, normal-appearing animals should be removed from the colony for laboratory tests. Serum samples should be obtained and tested for antibodies to murine viruses. Bacteria, mycoplasms, protozoa, and metazoa should be identified, if present. Tissues or organs should be examined histologically to determine the presence or absence of lesions.

Record Keeping - Daily records should be maintained on morbidity, mortality, and laboratory findings by room, species, and strain. This information should be reviewed weekly.

Parasitology - Microscopic examination of specimens obtained from fresh feces by concentration procedures and Scotch tape impressions of the perianal region from representative animals should be examined for the presence of parasitic ova.

At the time of sacrifice, in addition to routine methods described above, urine should be examined microscopically for nematode eggs, and the intestinal tract, cecum, and bladder opened and examined with appropriate magnification for internal parasites.

In addition, histologic examination of the tissue and organs will assist in determining whether selected protozoan or metazoan parasites are present. Special attention and selective stains are recommended for the lower respiratory tract and brain for Pneumocystic and Nosema, respectively.

Reference should be made to the Diagnostic Guide (Section I) (ILAR, 1971) and Disease Outlines (Section II) (ILAR, 1971) of "A Guide to the Infectious Diseases of Mice and Rats", for description of clinical and pathologic features of diseases plus appropriate diagnostic procedures. Positive and negative findings should be reported for each animal examined. It should be the responsibility of the laboratory supervisor to monitor the examination to assure its completeness and correctness.

3.5.3.10 Optimization of Facilities and Housing Conditions--

General Design - It is important wherever possible to provide access into the animal quarters from a "clean" corridor and egress via a "dirty" corridor. The traffic pattern should prohibit backflow from any area into a cleaner area. The animal quarters, in particular, should be well protected as well as corridors leading into them so as to prevent, for example, the movement of dirty cages down the "clean" corridor.

Temperature and Humidity - Each animal room or group of rooms with a common purpose should have individual temperature and humidity controls. The heating-cooling-ventilation system of the animal facility should be sensitive enough to permit adjustments within ± 1°C for any temperature within the range of 18°to 30°C (65°-85°F). A temperature of 23°C ± 1°C (74°F ± 2°F) should be maintained in all mouse and rat rooms. The optimum temperature for hamsters is 20°to 24°C. According to Federal regulations, the ambiant air temperature in rooms where these rodents are quartered should not be less than 16°C (60°F) or greater than 30°C (85°F). A relative humidity of 40% ± 5% should be maintained in all mouse and rat rooms. The relative humidity for hamsters should be 40 to 45%. An automatic recording and alert system should be used to monitor the ambient temperature and relative humidity in each animal room. An emergency power source should be available with a capacity sufficient to operate the air conditioning and light systems of the animal facility.

The temperature and relative humidity record charts for each 24-hour period throughout each bioassay test should be dated, signed, and filed for audit. The automatic devices for recording temperature and relative humidity should be recalibrated monthly. All pertinent data should be entered in a bound notebook and signed by the technical personnel who performed the work and by the supervisor. The alert and emergency power systems should be tested monthly and the results recorded.

Ventilation - Each animal room should have 10 to 15 fresh-air changes per hour without drafts. All air should be adequately filtered before entering and before discharge from the animal facility in order to lower the risk of transmitting viral or bacterial infections to the animals. HEPA (high efficiency particulate air) filters having a 99.9% to 95% efficiency for retaining particles of 0.3 micrometer or greater diameter are strongly recommended.

The general exhaust air from areas where chemical carcinogens are used is subject to Federal regulation. Recirculation of exhaust air from rooms where chemical carcinogens are used is not permitted. Air pressure should always be adjusted so that all animal rooms are slightly positive to the "dirty" corridor and negative to the "clean" corridor. Rooms bordering a single access corridor shall be kept under negative pressure with respect to the corridor. The animal facility and human occupancy areas should have separate ventilation systems.

A maintenance check on all mechanical ventilation equipment (air conditioner, blowers, fan motors, etc.) should be made monthly. Air intake and discharge filters should be inspected at least monthly and replaced when necessary. Air pressure of animal rooms with regard to entrance and egress corridors should be checked and adjusted, if necessary, each day. The number of fresh-air changes per hour in animal rooms should be monitored at least weekly and appropriate adjustments made when indicated. All data pertaining to the above must be entered in a bound notebook and signed by personnel involved.

Lighting - Housing quarters for laboratory animals should have ample light which is uniformly diffused throughout the area. Light intensity at the cage level shall be a minimum of 100 foot-candles. Examination and animal treatment areas should have a minimum light intensity of 125 foot-candles at the work surface. Continuous strip fluorescent lighting mounted flush in the ceiling is recommended. Fixtures must be properly sealed to prevent the harboring of vermin. Animal cages and other primary enclosures should be positioned so as to protect the animals from excessive illumination. A time-controlled system to provide a regular diurnal lighting cycle should be provided. Controls should be located in the main control room. Provisions must be made to provide hamsters with a lighting period of approximately 12 hours which is somewhat less than the optimum for other small rodents. Light switches should be located outside each room in both clean service and evacuation corridors, and lights should be serviced, if possible, via a crawl space or other method which does not necessitate entering the room. Convenience outlets should be waterproof, recessed in walls and partitions, and located a minimum of 0.6m (2 ft) above the floor.

Light intensity at cage level and at the work surface in animal examination and treatment areas should be determined weekly and adjusted if necessary. Instruments for determining light intensity should be calibrated monthly. The light cycle should be monitored regularly and adjusted if necessary to provide the optimal diurnal cycle for the species in question. The position of animal cages with respect to the light source should be checked regularly to make certain that animals are not subjected to excessive illumination. All test results and observations above must be entered in a bound notebook and signed by personnel involved.

Noise Control - Laboratory rodents, particularly mice, must be protected from noise, especially high-pitch noise (upper limits of human auditory range and beyond). Audiogenic strains must be maintained at very low noise levels. All noisy operations in the animal facility, such as cage and rack cleaning and washing, etc., must be carried out in an area separate from rooms where laboratory animals are housed. Animals should not be caged near incompatible species which disturb or distress them. Carts, trucks, racks, and other moveable equipment used in animal quarters should have rubber-tired casters and rubber bumpers. Concrete walls are preferred over metal or plaster construction to contain noise in animal quarters. Acoustical tile and similar materials should be used wherever possible to reduce the effect of "noise pollution" in animal rooms.

Evaluation of noise control practices should be included in all inspections of the laboratory animal facility and remedial measures instituted where necessary. A permanent record of these evaluations should be kept together with data recorded for the temperature, humidity, ventilation and lighting.

3.5.3.11 Drinking Water for the Animals--

In providing drinking water to the animals, watering bottles may be used although an automatic watering system is preferred. They must have an adequate supply of fresh and treated water ad libitum. Checks should be made to ensure that the water bottles are accessible to all animals and that sanitized water bottles, stoppers, and sipper tubes are supplied at least twice weekly. The animal care workers should routinely examine the watering device to assure its proper functioning.

Samples of the drinking water on a weekly basis as supplied into the animal quarters should be directly quality controlled by immediate chemical analysis (e.g., by gas chromatography, atomic absorption analysis, etc.) and by microbiologic culturing to screen for pathogenic microbes and viruses. Potential pathogens carried in the water should be killed or removed through appropriate treatment, such as sterilization, pasteurization, filtration, and/or chlorination. The methodology and standards described by Rand et al. (1975) should be the minimal criteria followed.

3.5.3.12 Laboratory Animal Feed and Bedding--

Feed should be accessible to all animals at all times. The feed containers should be durable and should be kept clean by sanitizing at least once a week, at which time remaining feed in these containers should be discarded. The containers should be mounted on the animals' cages so as to prevent or minimize contamination by excreta.

Date of manufacture and delivery of all feed supplies should be recorded upon receipt. Products delivered 90 days or more after manufacture should not be accepted.

Feed and bedding shall be stored in a clean area and protected from spoilage or deterioration and infestation or contamination by vermin. A continuous pest control program is essential. Containers should be stored off the floor on pallets, racks, or crates. The area should be physically separated from refuse areas.

Feed should be stored in receptacles with tightly fitting lids or covers which can be sanitized before reuse, or in original containers as received from the supplier. The storage area should be cool (10°C or less), dry and airy.

All supplies of feed and bedding as well as equipment in storage should be carefully protected against contamination by pesticides. No pesticides should be used inside buildings unless specifically agreed to by program management.

Temperature in the feed storage area should be recorded continuously by an automatic recording thermometer. Temperature recordings should be inspected daily and adjustments made when necessary to maintain a temperature of 10°C or less. All charts should be dated, signed, and filed for audit by program management.

The automatic temperature recorder should be recalibrated at least monthly and data recorded and signed by technical personnel performing the work.

All storage areas should be inspected weekly for the presence or evidence of vermin and appropriate action taken when necessary.

Feed in containers found open during inspections should not be used.

If possible, feed should be sterilized consistent with a disease control program. It is recommended that periodic sterilizer runs be monitored to assure that vegetative forms of microorganisms have been killed. This may be most easily accomplished by placing a filter paper strip impregnated with Escherichia coli in the center of load. The strip is then incubated in a suitable medium and examined for growth. Food may be held in a clean storage area until culture results are available.

Care must be taken to ensure that nutrients are not degraded or that the palatability of the feed is reduced. Random feed samples should be collected with each new batch of feed and analyzed in accordance with the Association of Official Analytical Chemists (AOAC, 1975). Samples (500-800 g) of each batch should be stored in a freezer for the duration of each bioassay so as to permit back-referencing against a control feed sample should this be required.

Feed that is older than 90 days may be considered unsatisfactory due to loss of essential nutrients.

As each new batch is received, samples of the feed should be chemically analyzed for pesticides, mycotoxin, industrial chemicals and biologic contaminants in accordance with the procedures described by the Association of Official Analytical Chemists. If unacceptable levels of contaminants are detected, the feed should not be used and a change in source might be investigated.

If pesticides are used in the animal facility, supplies of feed and bedding shall be analyzed at monthly intervals. Results of all analyses should be recorded and reported immediately to program management who will notify the bioassay laboratory of any lots unsuitable for use.

3.5.3.13 Vermin Control--

A safe and effective program for the control of insects, ectoparasites, avian and mammalian pests in and around the animal facility should be established and maintained under the supervision of a veterinarian or other qualified person.

The animal facility should be inspected weekly for the presence or evidence of vermin and remedial measures instituted if necessary. Results of inspections and remedial action taken should be recorded in a bound notebook, dated, and signed by inspector and supervisor.

Wild rodents and other vermin carry a variety of bacteria, viruses, and parasites which may be transmitted to experimental animals should they gain entrance to the facility. The population of wild rodents in the vicinity of animal buildings should be reduced or eliminated if possible.

3.5.3.14 Changing of Litter or Bedding, Changing of Laboratory Animal Cages and Disposal of Waste--

Cages should be program- and chemical-specific. They should be returned to the same chemical group and dose level to prevent test-chemical contamination.

Provision should be made for prompt removal and disposal of all food wastes from laboratory animal cages so as to minimize vermin infestation, odors, and disease hazards.

Measures must be taken to prevent molding, contamination, deterioration or caking of feed. Uneaten fruit or vegetable supplements must not be allowed to accumulate in animal cages.

Catch-pans for animals caged in exposure chambers should be cleaned and relined with new absorbent paper daily.

Animal cages should be inspected daily and litter or bedding changes as frequently as necessary, but not less than once per week, to comply with good animal laboratory practices.

Animal care personnel should be responsible for changing animals to sanitized cages with fresh bedding on at least a weekly basis.

Supervisors should monitor removal and disposal of all wastes containing chemical carcinogens or infectious agents to make certain that all procedures are in compliance with applicable Federal, State and local laws and regulations of the U.S. EPA.

Commercially available spore strips should be included in all autoclave loads of infectious waste and subsequently cultured to monitor the efficacy of the sterilization procedure.

Data pertaining to the disposal of infectious wastes or wastes containing chemical carcinogens must be entered in a bound notebook, dated, and signed by personnel involved and the supervisor.

Food and other wastes contaminated with known or suspected chemical carcinogens should be placed into separate plastic bags or other suitable impermeable containers for each carcinogen, closed, sealed, and labelled with both name of carcinogen and "DANGER -- CHEMICAL CARCINOGEN", before being transported to storage or disposal area. Final disposal should be in conformance with Federal, State and local laws, and with the NCI Office of Research Safety Regulation (NCI, 1976).

Wastes which are not contaminated with carcinogens or infectious agents may be disposed of at a public incinerator or burned at the facility. Incineration should comply with U.S. EPA regulations.

Infectious wastes should be autoclaved or rendered noninfectious by other effective measures before removal from the animal facility.

Waste disposal must comply with all Federal, State, and municipal laws, statutes, or ordinances.

3.5.3.15 Sanitation of Equipment and Supplies for Laboratory Animals--

Cages, racks, feeders, water bottles, catch-pans, exposure chambers, and certain ancillary equipment used in the EPA bioassay programs must be sanitized at specified intervals and before reuse.

Cages and racks should be washed with a suitable detergent at least weekly in a machine which provides at least one cycle of 82°C (180°F) water.

Soiled feeders should be soaked, if necessary, and then washed in a system that uses at least one cycle of 82°C water.

If water bottles are used, bottles, bottle stoppers, and sipper tubes must be washed in water of at least 82°C temperature. Stoppers and sipper tubes must be sterilized either by germicide treatment prior to washing or by boiling after washing.

Inhalation chambers shall be hosed down daily and sanitized weekly.

Catch-pans shall be cleaned and relined with fresh absorbent paper each day.

All sanitized cages, feeders, water devices, racks, catch-pans, and exposure chambers shall be inspected for physical cleanliness prior to reuse. Unsatisfactory items shall be resanitized.

Frequency of sampling of cages and other items for microbiological monitoring of the sanitization procedure will depend upon type of decontamination and level of protection desired. No gram-negative organisms should be detected, especially *Pseudomonas* spp., but sporeformers and heat-resistant non-pathogens will be found occasionally.

Detection of gram-negative organisms should result in an immediate shutdown of washing equipment and correction of the defect or institution of better room sanitization procedures, depending on probable source.

3.5.3.16 Disinfection and Sanitation--

- If an epizootic disease occurs among animals in quarantine or on test, the area shall be disinfected before use for new animals.

- Disinfectants for use in any part of the bioassay facility must be approved by EPA's program management.

- If formaldehyde gas is used, the room should be sealed and then treated by evaporating 500 ml of Formalin (37% solution of formaldehyde in water and stabilized with methyl alcohol) for each 27 m^3 of space. The temperature should be at least 21°C (70°F) and the relative humidity 75 to 80% during fumigation. The exposure period should be 24 hours.

- Disinfected animal rooms should not be reused until results of microbiological analyses indicate the absence of microorganisms pathogenic for humans and domestic animals. In this regard, the effectiveness of all disinfection procedures should be evaluated by sample swabbing of tables, benches, racks, walls and floor (at least one swab per area) and culturing (cell cultures, embryonated eggs, or laboratory animals). Acceptable diagnostic practices of the American Society of Clinical Pathologists or an equivalent organization should be used.

- Room and corridor floors, sinks, and pipes should be washed with a microbicidal solution weekly. Ceilings, walls, and partitions shall be treated in a like manner at regular intervals.

- After a room has been emptied of animals, all surfaces and fixed equipment should be washed with a microbicidal solution. Portable equipment for the room should be sanitized and/or sterilized, returned to the room, and the room and equipment fumigated. Paraformaldehyde is recommended for this purpose.

- The primary animal testing enclosures should be cleaned and sanitized often enough to prevent an accumulation of excreta, debris, dirt and harmful contamination.

- These enclosures should be sanitized by washing with hot water

(82°C) and soap or detergent, or by washing all soiled surfaces with a detergent solution followed by a safe and effective disinfectant, or by cleaning all soiled surfaces with live steam.

- All wastes should be collected and removed regularly and frequently in a safe sanitary manner. For example: highly infectious waste should be rendered noninfectious, by autoclaving or other effective means, before removing them from the animal facility.

3.5.3.17 Disposal of Dead or Sacrificed Animals and Their Tissues--

- All procedures involved in disposal of dead animals and tissues should be in conformance with Federal, State, and local laws and regulations pertaining to pollution control and protection of the environment.

- All dead animals should be subjected to full gross and histologic examination in accordance with EPA's bioassay program. Carcasses may be discarded immediately following necropsy and fixation of all required tissues needed for histopathology.

- Contaminated wastes, cleaning devices, and animal carcasses should be transported to the disposal area in a closed impermeable container and disposed of by methods approved by the EPA.

- Refrigerated storage should be available for holding dead animals until necropsy. The area should be separate from all other cold storage and should be used exclusively for refuse storage. The temperature should be kept below 7°C (45°F). The animals shall not be frozen.

- Supervisory personnel should monitor the disposal of all dead and sacrificed animals and tissues to make certain that all procedures are in accord with Federal, State, and local laws as well as with regulations of the EPA.

- Containers, liners, covers, etc., used in storage and disposal of sacrificed animals and tissues should be inspected during operations to maintain conformance with EPA's safety regulations.

3.5.3.18 Disposal of Radioactive Biologic Wastes--

- Radioactive biologic waste must be disposed of in accordance with applicable Federal and State regulations and licenses.

- A regular schedule for the collection of this waste should be set up.

- Leakproof disposable liners in waste cans must be used for burial or disposal of such radioactive waste.

- The storage area for radioactive waste should be physically separated from other storage facilities and animals.

- In some instances, the ordinary storage facilities may be used for holding the waste, if properly monitored, until removal for proper burial or disposal.

- Special shielding of the storage area may be required.

- Cage washing equipment should be of a type capable of decontaminating cage and accessories without accumulating radioactive waste. Machines should not recirculate the wash solution.

- A system of radiation monitoring must be instituted so as to prevent the spread of radioactive waste.

3.5.4 Bioassay Methods

The purpose of direct toxicity testing is to establish the potential harmfulness or safeness of single substances or mixtures of substances to humans and other animals via correlation of the biologic effects in test animals at different concentrations of the test substance (Hayes, 1975; Loomis, 1974). The ultimate response by a test animal is often an all-or-none effect (i.e., death, permanent neurologic damage, etc.), while some minimal lower concentration produces no measurable effect, each extreme varying from one animal to another. Such quantal or all-or-none responses differ from graded responses (i.e., body temperature, pulse or breathing rate changes) in that the latter can be continuously altered while the former cannot. The experimental determination of the range of dosages causing quantal effects in a group of animals is the basis of the quantal dose-response relationship that is often mathematically determined by probit analysis (Litchfield and Wilcoxon, 1949). Graded responses are analyzed using normal distribution theory.

Since the susceptibility of different individual animals and different species must be based on a common parameter of comparison, by convention the body weights of the test organisms are taken into account in dosing by establishing the mass of the organism as the basic unit of dosage (i.e., generally units of mg/kg or µg/kg of body weight). Dosage, the amount of test agent provided in relation to the weight of the test animals, should always be specified instead of the dose, which relates only the amount of agent given, independent of the animals' weights. It is often useful to denote the time dimensions with the dosage (i.e., mg/kg/hour), especially when it is necessary to repetitively dose these animals in the short interval of the acute bioassay.

3.5.4.1 Acute, Single, and Multi-Dosage Toxicity Bioassays--

The most widely accepted unit for expressing the results of acute, quantal, single-dose mammalian toxicity assays is the "1-ED50", the statistical estimate of the single dosage of test substance that produces the measured effect in 50% of a population of test species under stated assay conditions and routes of exposure. The measured quantal effect can be physiologic (i.e., sleep induction, total ataxia) or lethal. In the latter case, the unit is retitled the "1-LD50", the statistical median lethal dosage. Acute, quantal, multi-dose assays, generally performed within a 5-to 10-day interval in which dosages are administered daily, are less common but are sometimes necessary in order to test for an acute physiologic build-up of toxicants, especially substances which are lipidsoluble (i.e., vitamins A and D). In either the single-or the multi-dosage bioassays, randomly chosen groups of animals are established and each group is given one of a series of increasing dosages selected in such a way that the smallest dosage will produce the biologic effect in only a small number of each group receiving that dosage while the largest dosage yields the same effect in the majority of animals receiving the test substance at that level. Any effect measured in this way must be an all-or-none response and can thus be easily statistically analyzed by Litchfield and Wilcoxon's probit analysis technique. An outline of a sample protocol is given in the following pages.

EXAMPLE: ACUTE IN VIVO TEST IN RODENTS

Purpose of Study

- Mammalian acute toxicity determination

Materials

- Young adult rats (approximately 250 g each) can be purchased from the supplier.

Design of Experiment

- It is recommended that a two-step approach be taken to initial acute in vivo toxicology evaluation of unknown compounds. These two approaches are: The quantal (all-or-none) response and the quantitative (graded) response. Normally, the quantal assay is used to determine the necessity to carry out the quantitative assay.

Quantal Assay

- Number of Animals: Five male and five female rats.
- Quarantine Period: A minimum of five days.
- Dose: A single dose of 10 g per kg of test material undiluted if a liquid, diluted with a biologically inactive solvent if a solid. A control is required.
- Route of Administration: By gavage. If no mortality occurs in the quantal study, no further work need be done on the test substance. If a single animal in this study dies in the 14 day observation period, then a quantitative study will be performed.

Quantitative Assay

- Number of Animals: One hundred animals equally divided by sex will be used for this study.

- Quarantine Period: 7 days.
- Health of Animals: Must be good when ready to be experimented upon.
- Randomization: The animals will be randomly divided into five groups of five male and five female animals per group.
- Test Material: Selected as in quantal study.
- Dosing: 3.0, 1.0, 0.3, 0.1 g per kg and a control. This dosage schedule will be selected depending greatly on the results of the quantal study in regard to the numbers of animals that died and severity and type of signs. Require randomization.
- Route of Administration: By gavage.

Conduct of Experiment

QUALITY CONTROL -- Establish regular audits of performance throughout the experiment.

Quantal Assay

- Observation:
  (1) Immediately following administration of test material and at frequent intervals during the first day, observe all toxic signs or pharmacological effects indicated in Table 1.
  (2) Score the frequency and severity of the signs.
  (3) Pay particular attention to time of onset and disappearance of signs.
  (4) Make daily observation on all animals through the test period (14 days).
  (5) Investigate further the test materials which produce harmful effects in vivo and do not result in deaths.
  QUALITY CONTROL -- Signing and witnessing of all records.
- Pathology: At end of observation period, kill all surviving animals and perform necropsies. Likewise, perform necropsies on all animals that die during the course of the assay.
  QUALITY CONTROL -- Signing and witnessing of all data collected.

Quantitative Assay

- Observation: Same as indicated in quantal assay.
  QUALITY CONTROL -- Signing and witnessing of all records.
- Pathology: Same as indicated in quantal assay.
  QUALITY CONTROL -- Same as above.

Data Analysis

Quantal Assay

- Should no mortality occur in the quantal study, the LD50 should be reported

as greater than 10 g per kg.
QUALITY CONTROL -- Use statistical expertise in analysis of results.

Quantitative Assay

- Calculate LD50 by methods of Horn (1956) or other reliable methods
  QUALITY CONTROL -- Use statistical expertise in analysis results.
- If the data are not suitable for a precise LD50 calculation, i.e. total mortality occurs in the lower dosage level, make an estimate of the LD50 or express LD50 as greater than 3 g per kg or less than 0.1 g per kg.
  QUALITY CONTROL -- Use standard statistical techniques at all times.
- Depending on the results of the assay of higher dosages, lower dosage or another series at intermediate dosages may be a necessity.

Reporting Data

- The report should include: (1) a statement of methods, (2) the results obtained, (3) a statement of conclusion.
  QUALITY CONTROL -- Adopt a good system for review and publication of reports.

References

Horn, H. 1956. Simplified LD50 (or ED50) calculations. Biometrics Vol. 12, pp. 311-322.

The discussion in this example is principally derived from the following report:

Duke, K.M., M.E. Davis, and A.J. Dennis. 1977. IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests for Pilot Studies. EPA-600/7-77-043, April 1977.

TABLE 1. PHYSICAL EXAMINATIONS IN ACUTE TOXICITY TESTS IN RODENTS

Organ System	Observation and Examination	Common Signs of Toxicity
CNS and somatomotor	Behavior	Change in attitude to observer, unusual vocalization, restlessness, sedation
	Movements	Twitch, tremor, ataxia, catatonia, paralysis, convulsion, forced movements
	Reactivity to various stimuli	Irritability, passivity, anaesthesia, hyperaesthesia
	Cerebral and spinal reflexes	Sluggishness, absence
	Muscle tone	Rigidity, flaccidity
Autonomic nervous system	Pupil size	Myosis, mydriasis
	Secretion	Salivation, lacrimation
Respiratory	Nostrils	Discharge
	Character and rate of breathing	Bradypnoea, dyspnoea, Cheyne-Stokes breathing, Kussmaul breathing
Cardiovascular	Palpation of cardiac region	Thrill, bradycardia, arrhythmia, stronger or weaker beat
Gastrointestinal	Events	Diarrhea, constipation
	Abdominal shape	Flatulence, contraction
	Feces consistency and color	Unformed, black or clay colored
Genitourinary	Vulva, mammary glands	Swelling
	Penis	Prolapse
	Perineal region	Soiled
Skin and fur	Color, turgor, integrity	Reddening, flaccid skinfold, eruptions, piloerection
Mucous membranes	Conjunctiva, mouth	Discharge, congestion, hemorrhage cyanosis, jaundice
Eye	Eyeball	Exophthalmus, nystagmus
	Transparency	Opacities
Others	Rectal or paw skin temperature	Subnormal, increase
	Injection site	Swelling
	General condition	Abnormal posture, emaciation

3.5.4.2 Subacute Toxicity Bioassays--

Subacute toxicity testing involving protracted dosing has been performed, more or less by convention, up to one-tenth of an experimental animal's lifespan. For rats a 90-day interval is considered adequate, while dogs are tested for a 1-year period, in each case with daily dosing. Boyd and Boyd (1962) appear to have been the first to report subacute toxicity in the form of a

90-LD50 (i.e., a 90-day median lethal dosage). Boyd (1968) later pointed out that subacute tests using rats would be reduced to 70 days (i.e., 70-LD50) essentially without loss of important information. The results of 90-day studies not only in rats but also in dogs have been shown by Weil and McCollister (1963) to be similarly comparable to corresponding lifetime studies in these species for a wide variety of compounds. As reviewed by Hayes (1975) the 90-LD50 (or 90-ED50) is statistically comparable to the 1-LD50 (or 1-ED50) in that the dosage expressed in logarithms is linearly related to the percentage biologic effect expressed as probits.

An example of a subacute bioassay protocol is given below.

EXAMPLE: MAMMALIAN SUBACUTE BIOASSAY

Purpose

The purpose of subacute studies is to determine what cumulative effects might become manifested during prolonged subacute exposure to the test substance and to provide pilot chronic study information through one-tenth of the species' lifespan prior to performing a longer term (chronic) bioassay.

Design of Experiment

The preferred method (Zbinden, 1973) of assessing subacute toxicity is to challenge both sexes of at least two species of animals (one rodent and one non-rodent) with diets dosed with at least three levels of test agent, and to compare the biologic responses of these test animals after prolonged exposure with the responses in control animals fed the same but undosed diet over the same interval. The Maximum Tolerated Dosage, or MTD, estimated to cause zero deaths during the preceding acute animals study should be the highest level of exposure incorporated into an average dietary feeding portion (Sontag et al., 1976). Thus, the concentration of test substance at the highest test level should afford the animal not more than 1-MTD in each average daily feeding portion (e.g., 1-MTD per 25.0 g of feed per rodent, assuming it has been demonstrated that this amount of feed is the average amount consumed by each rodent). At least two other lower dietary test levels (e.g., 1/3-MTD and 1/9-MTD, or 1/5-MTD and 1/25-MTD) also should be bioassayed with separate randomly picked groups of animals.

Summary of Experiment Design

Species	Group	No. Females	No. Males	Dosage	Duration
Rat	1	20	20	Control	90 days
"	2	20	20	MTD	90 days
"	3	20	20	1/3 MTD	90 days
"	4	20	20	1/9 MTD	90 days
Dog	1	5	5	Control	1 year
"	2	5	5	MTD	1 year
"	3	5	5	1/3 MTD	1 year
"	4	5	5	1/9 MTD	1 year

Route of Administration of Test Substance

- Dosing of test animals on a long-term basis (e.g., 90 days to 1 year) is feasible only through the animals' diet, since other routes such as intramuscular injection or stomach tube gavage are less convenient and afford more trauma to the animals.
- Accurate and up-to-date record keeping of body weight changes and amount of food ingested are essential for later interpretation of comparative mortality and pathology data between control and test populations.
- At the end of the prescribed dietary exposure period, it is required that surviving rodents be fed an uncontaminated diet for an additional 2 weeks or longer while surviving dogs be given uncontaminated feed for an added 4 weeks or longer prior to sacrifice, necropsy, and histologic examination.

Exposure Schedule

- The test substance should be ingested daily by all test animals for the duration of the bioassay as described in the above experimental design.

Design Outline

- Two species of mammals each consisting of four groups (20 rodents per group or 5 dogs or cats per group); one group serves as the negative (vehicle) control, while the remaining three groupings ingest geometrically decreasing dosages, the highest being the MTD; rodents observed a minimum of 2 weeks further on the uncontaminated diet following the full exposure duration; dogs further studied a minimum of 4 weeks.
  QUALITY CONTROL -- Same as that outlined for acute toxicity bioassay. Analyze periodically freshly mixed diet samples and maximally aged diet samples, as used in the study to assure stability of the toxicant.

Observations and Bioassays

- Twice daily records should be kept on all animals during and after the exposure with respect to relevant clinical signs (see list below), all function tests and histopathologies, morbidity rate, necropsy observations, etc.; all survivors including controls should also be examined clinically (see list below), by histopathology and by necropsy for comparison with non-survivors.
  QUALITY CONTROL -- Same as that outlined for acute toxicity bioassay.

Clinical Laboratory Studies in Subacute Bioassays

- The following clinical laboratory assays should be performed on a minimum of 5 animals of both sexes of both species from the control and all the test groups (selected randomly in the cases of survivors) on a scheduled basis (i.e., at 0,45 and 90 days and at termination for rodents, and at 0, 26, and 52 weeks and at termination for dogs or cats, where the 0th day or week immediately precedes exposure of the test groups).

Hematology

Hematocrit
Hemoglobin
Erythrocyte count
Total leukocyte count
Differential leukocyte count
Reticulocyte count

Clinical Chemistries

Blood sugar
Blood urea nitrogen
Alkaline phosphatase
Serum glutamic oxaloacetic transaminase
Serum glutamic pyruvic transaminase

SMAC determinations will be made at termination, including in addition to the above:

Creatinine
Cholesterol
Uric acid
Total protein
Creatine phosphokinase
Albumin
Calcium
Phosphorus
Globulin
CO_2
Potassium
Total bilirubin
Lactic acid dehydrogenase
Iron
Triglycerides
Sodium chloride

Urinalysis

pH
Ketones
Glucose
Albumin
Specific gravity

Autopsy and Terminal Necropsy Examinations

- Gross examination should be done as called for in the acute bioassay.

Histopathologic Examinations

- Histopathologic examinations should be performed on selected tissues and organs.

Records and Reports

- Records will be maintained on:

  Sampling assays
  Daily observations
  Pathology
  Feed consumption
  Mortalities
  Body weights
  Necropsies

Changes or Revisions

- Any changes or revisions of this approved protocol will be documented, signed by the study director, dated, and maintained with this protocol. The sponsor will be notified prior to any revision of this protocol.

3.5.4.3 Chronic Toxicity Bioassays--

It is not valid to assume that chemicals which produce a finite toxicity in one month will, at one-half the concentration, produce the same toxicity in two months. Consequently, if human exposure has been or will be protracted over several months or years, then animals should be bioassayed for equivalent lengths of their lifespans by comparable routes of administration and at comparable as well as excessive dosages. Otherwise, extrapolation of these animal results to mankind would be invalid.

In order to assess the potential long-term hazards of low levels of suspected toxic substances in our food, water, environment, etc., the results of acute and subacute animal studies must be extended in chronic, low-level exposure bioassays in other animals so as to provide sufficient data to permit an intelligent evaluation of all possible dangers at a minimum cost in time and money. In general, chronic mammalian toxicity studies are directed at investigating the chemical induction of specific phenomena such as carcinogenesis, long-term cytotoxicity, mutagenesis, and teratogenesis and reproductive retardation. This subsection, however, will be limited to discussion of currently accepted techniques of evaluating the carcinogenic potential of chemical substances and mixtures.

At present, the assessment of the carcinogenic potency of a substance is based on the subjective judgment of a qualified pathologist making histopathologic evaluations of tissues taken from exposed or treated animals. The experience of the pathologist with tissues from many different animal species under various test conditions is critical since there is no objective means for correlating observed long-term effects with chronic treatments or exposures at present, other than by direct chemical analysis for the tested substance in target tissues and organs. The histopathologic observer must be able to recognize the interfering effects of concurrent infections, intracage fighting, numbers and types of "spontaneous" tumors, chronic degenerative diseases, dietary deficiencies or any other condition, unrelated to the chronic exposure or treatment, that may bias the carcinogenicity results. The answers, at present, cannot be found in statistical data alone, but must be combined with pathologic evaluations before a safe exposure limit level for mankind can, if possible, be established.

Because of the duration, effort, and expense involved in conducting chronic toxicity studies in mammals, any preliminary effort is well spent when expended in selecting the most pertinent dosage levels, the best conditions of animal husbandry and hygiene, the accuracy of the dietary exposures or chronic dermal or inhalatory treatments, quality of mixing vehicle, avoidance of bias from contaminants in water, air and food, etc. To control contaminant interferences, poor hygiene, etc., the National Cancer Institute has recommended (Sontag et al., 1976) that a second set of untreated control animals, independent of the vehicle control animals, be incorporated into the overall bioassay. These untreated animals should, moreover, be housed in a separate room with a separate air and water supply so as to reduce inadvertent exposure to the test agent. These design controls have been summarized in outline form in the sample protocol on the following pages.

EXAMPLE: MAMMALIAN CHRONIC BIOASSAY

Purpose

Carcinogenicity determination.

Summary of Experimental Design

Species†	Group	No. Females	No. Males	Dosage*	Duration
Mouse	1	60	60	Control-A	30 months
"	2	60	60	Control-B	30 months
"	3	60	60	MTD	30 months
"	4	60	60	1/3 MTD	30 months
"	5	60	60	1/9 MTD	30 months
Dog	1	30	30	Control-A	48 months
"	2	30	30	Control-B	48 months
"	3	30	30	MTD	48 months
"	4	30	30	1/3 MTD	48 months
"	5	30	30	1/9 MTD	48 months

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* Control-A is the untreated separated control group; Control-B is the vehicle-treated control group; MTD is the maximum tolerated dosage as determined in the preceding subacute bioassay; 1/3 and 1/9 MTD are fractions of the MTD.

† Dogs or cats may be required because some of their metabolic and physiologic characteristics of interest may be closer to humans than those of rats or mice.

Chronic Exposure Schedule and Routes of Administration

- Three dosage levels are recommended (Grice and Da Silva, 1973) in chronic bioassays beginning with the MTD (maximum tolerated dosage) as the highest level and geometric reductions in dosage used at two lower levels.

- The test substance should be ingested daily by all test animals for the duration of the bioassay as described in the above summary. Dermal dosing of the test agent in a chronic bioassay might also be conducted in a similar manner, but only for those substances where cutaneous contact is the main route of human exposure.

- A minimum of 60 smaller mammals (rodents, rabbits, hamsters, guinea pigs, etc.) per sex per test and control group is required for observing a minimum of a 5% incidence of chronic toxic effects with a confidence probability of 0.95 (Zbinden, 1973).

- A minimum of 30 larger mammals (dogs, cats) per sex per test and control group is required for observing a minimum of a 10% incidence of chronic toxic effects with a confidence probability of 0.95 (Zbinden, 1973)

- The test agent should be given for a length of time sufficient to yield the maximum response in the test animals. For this reason it is recommended that rodents and rabbits be tested for as long as 30 months, and dogs and cats for as long as 48 months. When 20% survival of test animals and/or 20% tumor-free incidence are reached, however, consideration must be given to earlier termination of the investigation.

- When exposure is through the feed or drinking water on a daily basis, the amount of the test substance consumed must be quantified via measurement of the food or water intake. Body weights must also be recorded at least on a weekly basis to determine if eating patterns have been altered.
- If more than 20% mortality occurs in the first 18 months among the smaller mammals (mice, rats, hamsters) serving as vehicle-treated controls, consideration should be given to declaring this particular trial invalid. Similar invalidation should be considered if 20% mortality results in the first 30 months among the larger mammals (dogs, cats).
- No recovery period should be permitted; instead, all survivors should be sacrificed at termination for necropsy and histopathologic examination.

Design Outline

- Two species of mammals of each sex and each consisting of 5 groups (60 rodents per group or 30 dogs per group); one group serves as untreated, separated controls; another group serves as vehicle-treated controls; remaining groups are dosed with geometrically decreasing amounts of test agent, the highest being the MTD via mixing into feed or drinking water. QUALITY CONTROL -- Same as that outlined in acute toxicity bioassays especially periodic analyses of the feed and water for extraneous or contaminating substances which may interfere.

Observations and Bioassays

- Twice daily recordings of morbidity rate and autopsies, if any; daily recordings of quantities of water and feed ingested; weekly recordings of body weight and general health observations; monthly recordings of relevant clinical signs and function tests (see list below); all recordings of gross anatomical and histopathologic investigations or autopsies and final necropsies as occur for all non-survivors and for all animals sacrificed at termination of each chronic study.
  QUALITY CONTROL -- Same as that outlined in acute toxicity bioassays especially with respect to record keeping, signing and witnessing of notebooks, "blind status" of examining histopathologist, and survival of at least 80% of control animals within first 18 months for rodents and 30 months for dogs.

Clinical Laboratory Studies in Chronic Bioassays

- Clinical laboratory assays should be performed as in the Subacute Bioassay.

Autopsy and Terminal Necropsy Examinations

- Gross examinations should be made as in the Acute Bioassay.
- If mortality in any test group after 18 months for rodents or 24 months for dogs or cats approaches 50%, consideration should be given to early termination of the bioassay.

Histopathologic Examinations

- Histopathologic examinations will be performed on selected tissues and organs.

Records and Reports

- Records will be maintained on:

Sampling assays
Feed consumption
Body weights
Daily observations

Mortalities
Necropsies
Pathology

- In addition to the final report, interim reports may be made available to the sponsor if required. The frequency of such reports will be determined prior to study initiation. The report will be a complete scientific presentation of results and conclusions.

Changes or Revisions

- Any changes or revisions of this approved protocol will be documented, signed by the study director, dated, and maintained with this protocol. The sponsor will be notified prior to any revision of this protocol.

3.5.4.4 Chronic Inhalation Study in Rats with In Utero Exposure--

EXAMPLE: EFFECT ON LITTER OF CHRONIC INHALATION BY PARENTS BEFORE BREEDING

Purpose of Study

Determination of effects on reproduction process
Chronic toxicity determination
Carcinogenic evaluation

Design of Experiment

- Phase I - Reproduction: 90 male and 180 weanling Fischer rats, 70 to 90 g in weight.

Group No.	No. of Animals Male	Female	Dose Levels
1	15	30	Control
2	15	30	1/100 MTD
3	15	30	1/30 MTD
4	15	30	1/10 MTD
5	15	30	1/3 MTD
6	15	30	MTD

- Breed to obtain 25 litters per group (estimate 85% of bred females will litter). At weaning, randomly select 3 males and 3 females from each litter for Chronic Toxicity Phase.

Phase II - Chronic Toxicity and Carcinogenicity

Group No.	No. of Animals Male	Female	Dose Levels
1	75	75	Control
2	75	75	1/100 MTD
3	75	75	1/30 MTD
4	75	75	1/10
5	75	75	1/3
6	75	75	MTD

Route of Administration

- Inhalation exposure in stainless steel chambers with effective exposure areas of 5.5 x 5.5 x 6.0 feet.

Exposure Schedule and Subsequent Handling

- Phase I - Reproduction
  - Expose parent generation in individual cages 23 hours per day, 7 days per week for approximately 100 days or for at least 60 days prior to breeding.
  - At end of exposure period, one male and two females will be placed in each breeding cage for one week.
  - Females will then be placed in individual cages and allowed to litter and nurse their offspring for 28 days. The litters will be reduced to eight pups, half of each sex, on day 4 of lactation.
- Phase II - Chronic Toxicity

- One week after weaning (day 28) of the first litter, all excess rats (above 8 from each of 25 litters) will be discarded and remaining rats will be identified by ear tag. This will be considered Week 0 for recording purposes. The actual data of birth and heritage of each rat retained for the chronic study will be recorded for possible further evaluation. These rats will be individually housed.

Conduct of Experiment

QUALITY CONTROL -- At experimental design stage, advice of a statistician should be sought on group size and on methods of statistical analysis to be used.

QUALITY CONTROL -- Hold in quarantine for 1 week.

- Assign animals to study group as removed from shipping crates.
  QUALITY CONTROL -- Use randomization procedures.

- Identify animals by cage, group and individually.
  QUALITY CONTROL -- Achieve an equivalent mean body weight between groups.
  QUALITY CONTROL -- Use ear tags and durable cage markers.
  QUALITY CONTROL -- Accurately determine body weight and food consumption.

- House individually.

- Supply food and water ad libitum.
  QUALITY CONTROL -- Use commercial rodent ration.

- Analyze basic laboratory diet for contaminants PBB's and PCB's, antibiotics, estrogens, aflatoxins, lead, arsenic and mercury and nutritional content.
  QUALITY CONTROL -- Selected frequency, rejection of contaminated feed, and inter- and intra-lab control tests.

- Analyze water for heavy metals and coliforms.
  QUALITY CONTROL -- Quarterly.

- Generate atmospheres by method appropriate to the test material. For volatile liquids generate high concentrations by passing compressed air through the liquids at constant rates. Reduce to dilution with filtered warm air drawn through the chambers which operate under negative pressure.
  QUALITY CONTROL -- Monitor continuously; prior to exposure of animals calibrate the monitoring equipment (such as hydrocarbon analyzer) with the substance being tested. The range of calibration points will encompass the selected dosage levels. Aliquots of the test substance will be introduced into large gas sampling bottles of known volume. After vapor concentration reaches equilibrium the aliquot will be introduced into the analyzer.
  QUALITY CONTROL -- Analyzer should be equipped with 10-point timed solenoid sampling system: 1 to 8, level of substance in eight chambers; 9, room atmosphere; 10, combined stack effluent.
  QUALITY CONTROL -- Sample four times each day for 10 minutes per sampling point.

QUALITY CONTROL -- Adjust flow as required.

Observations and Tests

- Phase I - Reproduction: The following information will be collected:

  1. Parent body weights and food consumption at 1, 4, and 8 weeks of treatment.
  2. Observations on parents tabulated weekly.
  3. Live and dead pups and external appearance at birth.
  4. Number, sex, appearance, and individual weight of pups at day 4.
  5. Number, sex, appearance, and individual weight of pups at day 28.

  Any abnormal pups will be killed and prepared for examination by fixing in Boulin's solution or by clearing for skeletal examination. The males will be killed and gross necropsy performed after weaning (day 28). The uteri of nonpregnant females will be inspected for resorption sites. At 100 days of age, one male and one female from each litter (total 25 males and 25 females) will be sacrificed. Organ weights will be recorded for each rat as at termination.
  QUALITY CONTROL -- Good form design.
  QUALITY CONTROL -- Signing and witnessing of all records.

- Phase II - Chronic Toxicity: Individual body weights and food consumption will be recorded at monthly intervals. Observation of gross signs of toxicity, pharmacologic effects, and the incidence, size, and location of tumors will be recorded at the same intervals. All animals will be observed daily for mortality. Starting at 18 months and continuing until termination, mortality observations will be made twice daily. Necropsies will be performed on all animals that die during the course of the study, and tissues will be taken. Should an animal be moribund and not anticipated to live to the following day, it will be killed, necropsy performed, and tissues taken and preserved as described below for terminal examinations.
  QUALITY CONTROL -- Good form design.
  QUALITY CONTROL -- Signing and witnessing of all records.

Clinical Laboratory Studies

- The following clinical laboratory studies will be performed on five male and five female animals from the control and each test group (selected from among those individually housed):

  - Hematology - at 26 and 52 weeks, and termination includes:

Hematocrit	
Hemoglobin	Total leukocyte count
Erythrocyte count	Differential leukocyte count
	Reticulocyte count

  - Blood Chemistry - at 26 and 52 weeks, includes:

Blood sugar
Blood urea nitrogen
Serum glutamic oxaloacetic transaminase
Alkaline phosphatase
Serum glutamic pyruvic transaminase

SMAC determinations will be made at termination, including in addition to the above:

Creatinine
Cholesterol
Uric acid
Total protein
Creatine Phosphokinase
Sodium
Phosphorus
Globulin
Total bilirubin
Albumin
Calcium
Iron
Chloride
CO_2
Potassium
Lactic acid dehydrogenase
Triglycerides

- Urinalysis - Using pooled samples from the five rats per group per sex at 26 and 52 weeks, and termination includes:

pH
Glucose
Ketones
Specific gravity
Albumin

Termination and Postmortem Examination

- The study will be terminated at 30 months or 20% survival, whichever comes first, and all surviving rats will be killed and necropsied. The weights of the following organs will be recorded for each rat and the organ weight and body weight ratios will be calculated:

heart
liver
spleen
kidneys
adrenal glands
testes (with epididymides)
lungs
brain
thymus

Histopathologic Examination

- Histopathologic examination will be performed on all of the following tissues from all rats in the two highest dosage groups having adequate survival, all controls, and those dying during the course of the study. Three target organs from all animals in the remaining groups will be examined histopathologically. Selected body tissues and organs will be analyzed for content of the toxicant and/or biotransformation products.

- Appropriate samples of each will be preserved in 10% neutral formalin:

all gross lesions
brain (cerebrum, cerebellum, brainstem)
spinal cord (2 sections)
eye
pituitary
salivary gland
esophagus
stomach
small intestine (duodenum, ileum, jejunum)
large intestine (colon, cecum)

- heart
- thymus
- thyroid
- lungs (with mainstem bronchi)
- trachea
- spleen
- bone (with marrow)
- lymph nodes (2)
- skeletal muscle
- adrenal glands
- pancreas
- liver
- kidneys
- urinary bladder
- ovaries/testes
- prostate
- uteri (corpus, cervix)
- skin (mammary area)

A peripheral blood smear will be made and maintained for possible future examination in the event that other histopathologic findings suggest leukemia or other blood-related alterations.

Records and Reports

- Records will be maintained on:

    - sampling assays
    - feed consumption
    - body weights
    - daily observations
    - mortalities
    - necropsies
    - pathology

- In addition to the final report, interim reports may be made available to the sponsor if required. The frequency of such reports will be determined prior to study initiation. The report will be a complete scientific presentation of results and conclusions.

Changes or Revisions

- Any changes or revisions of this approved protocol will be documented, signed by the study director, dated, and maintained with this protocol. The sponsor will be notified prior to any revision of this protocol.

Approval of Protocol

Date:______________________________ Sponsor:____________________________

Date:______________________________ Study Director:______________________

EXAMPLE: 24 MONTH INHALATION STUDY IN MICE

Purpose of Study

Carcinogenic effects determination

Design of Experiment

- 300 male and 300 female young B6C3F1 strain mice less than 20 g in weight.

Group No.	No. of Animals Male	No. of Animals Female	Dose Levels
1	50	50	Control
2	50	50	1/8 MTD
3	50	50	1/4 MTD
4	50	50	1/2 MTD
5	50	50	3/4 MTD
6	50	50	MTD

- All animals will be housed by sex and dosage, five animals per cage.

Route of Administration

- Inhalation exposure in stainless steel chambers with effective exposure areas of 5.5 x 5.5 x 6.0 feet.

Exposure Schedule

- Exposure for 23 hours per day, 7 days a week.

Conduct of Experiment

QUALITY CONTROL -- Advice of a statistician should be sought on group size and on methods of statistical analysis to be used.
QUALITY CONTROL -- Hold in quarantine for 1 week.

- Assign animals to study group (as removed from shipping crates) following quarantine.
  QUALITY CONTROL -- Use randomization procedures.
- Identify animals by cage, group, and individually.
  QUALITY CONTROL -- Achieve an equivalent mean body weight between groups.
- House animals individually.
  QUALITY CONTROL -- Use ear tags and durable cage markers.
  QUALITY CONTROL -- Accurately determine body weight and food consumption.
- Supply food and water *ad libitum*.
  QUALITY CONTROL -- Commercial rodent ration.
- Analyze basic laboratory diet for contaminats; PBB's and PCB's; antibiotics,

estrogens, aflatoxins; lead, arsenic, mercury; nutritional content.
QUALITY CONTROL -- Selected frequency.
QUALITY CONTROL -- Reject if contaminated.
QUALITY CONTROL -- Inter- and intra-lab control tests.

- Analyze water for heavy metals and coliforms.
  QUALITY CONTROL -- Quarterly.

- Generate atmospheres by method appropriate to the test materials. For volatile liquids generate high concentrations by passing compressed air through the liquids at constant rates. Reduce to dilution with filtered warm air drawn through the chambers which operate under negative pressure.
  QUALITY CONTROL -- Monitor continuously; prior to exposure of animals calibrate the monitoring equipment (such as hydrocarbon analyzer) with the substance being tested. The range of calibration points will encompass the selected dosage levels. Aliquots of the test substance will be introduced into large gas sampling bottles of known volume. After vapor concentration reaches equilibrium the aliquot will be introduced into the analyzer. Analyzer should be equipped with 10-point automatically timed solenoid sampling system: 1 to 8, level of substance in eight chambers; 9, room atmosphere; 10, combined stack effluent.
  QUALITY CONTROL -- Sample four times each day for 10 minutes per sampling point.
  QUALITY CONTROL -- Adjust flow as required.

Observations and Tests

- Individual body weights and the number of survivors will be recorded for each group weekly until the weights of the animals stabilize (13 weeks). Body weights thereafter will be done on a monthly basis. Feed consumption for each cage group will also be recorded at the same intervals.

- All animals will be observed at least every 8 to 10 hours for deaths and morbidity. Observations of gross signs of toxicity, pharmacologic effects and incidence, size, and location of tumors will be recorded at weekly intervals. Should an animal appear moribund, have an obvious lesion, or a grossly evident tissue mass, it will be housed separately to prevent cannibalism.

- Should an animal be moribund and not anticipated to live to the following day, it will be killed, a necropsy performed, and the tissue taken and preserved. Necropsies will be performed on all animals that die during the course of the study, and the tissues will be taken and fixed.

Termination and Postmortem Examination

- The study will be terminated at 24 months and all surviving mice will be necropsied. If the mortality in any dose sex group between 18 and 23 months approaches 50%, consideration should be given to early termination of the study.

Histopathologic Examination

- Histopathologic examination will be performed on all of the following tissues from all mice in the two highest dosage groups having adequate survival, all controls, and those dying during the course of the study. Three target organs from all remaining groups will be examined histopathologically. Appropriate samples of each will be preserved in 10% neutral formalin:

all gross lesions
brain (cerebrum, cerebellum, brainstem)
spinal cord (2 sections)
eye
pituitary
salivary gland
heart
thymus
thyroid
lungs (with mainstem bronchi)
trachea
spleen
bone (with marrow)
lymph nodes (2)
esophagus
stomach
small intestine (duodenum, ileum, jejunum)
large intestine (colon, cecum)
adrenal glands
pancreas
liver
kidneys
urinary bladder
ovaries/testes
prostate
uteri (corpus, cervix)
skin (mammary area)
skeletal muscle

- A peripheral blood smear will be made and maintained for possible future examination in the event that other histopathologic findings suggest leukemia or other blood-related alterations.

Records and Reports

- Records will be maintained on:

sampling assays
feed consumption
body weights
daily observations
mortalities
necropsies
pathology

- In addition to the final report, interim reports may be made available to the sponsor if required. The frequency of such reports will be determined prior to study initiation. The report will be a complete scientific presentation of results and conclusions.

Changes or Revisions

- Any changes or revisions of this approved protocol will be documented, signed by the study director, dated, and maintained with this protocol. The sponsor will be notified prior to any revision of this protocol.

Approval of Protocol

Date:________________________ Sponsor:________________________

Date:________________________ Study Director:____________________

3.5.5 Gross Observations

3.5.5.1 Mortality--

Death of a whole animal is generally defined (Gove, 1976) as "the ending of all vital functions without possible recovery". These vital functions or signs consist of voluntary movements, breathing, blood pulse, eye blinking and other involuntary reflexes, righting reflex, and electroencephalographic activity. As a quality control procedure, it may be suggested that an explicit definition should be made of the criteria of declaring occurrences of mortality in each bioassay protocol. Requirements should also be established as to the technical backgrounds and/or licenses of those investigators who shall function to certify deaths and perform autopsies, sacrificing of animals and necropsies. It has been recommended (Hinkle, 1977) that veterinarians be accredited by the American College of Laboratory Animal Medicine (ACLAM) and that animal laboratory technicians be accredited by the American Association for Accreditation of Laboratory Animal Care (AALAC) prior to establishing bioassay programs.

In a similar vein, the criteria for cellular death, necrosis, and autolysis ought to be delineated in each proposal for a bioassay investigation where gross and microscopic pathologic examinations are to be made. Here also, the need for qualified personnel, particularly pathologists and technicians certified by the American Society of Clinical Pathologists (ASCP), the College of American Pathologists (CAP), the American Association of Experimental Pathologists (AAEP), the American Industrial Hygiene Association (AIHA) or the above ACLAM, should be apparent.

3.5.5.2 Body Weight, Growth, and Nutrition--

Routine evaluation of the growth and development of large numbers of animals receiving a test compound has been a crucial means of predicting possible toxic effects, especially in subacute and chronic studies (Barnes and Heath, 1964: McLean and McLean, 1969; Case et al., 1976). The quality control aspects to be applied to these studies should, of course, involve a means of certifying the accuracy of equipment used in measuring the animals' weights. To this end, the following recommendations are made:

- All animals used in an EPA bioassay program should be weighed individually at these times: At time of receipt, at time of assignment to treatment groups, and periodically during the actual bioassay.
- Weights should be determined to the nearest gram using an appropriate animal weighing scale.
- Balances employed for determining animal weights should be recalibrated against NBS approved standards at least monthly and calibration data recorded in a bound notebook and signed by the responsible personnel.
- Supervisors should be responsible for making certain that all animal weights are accurately determined and recorded to insure validity of the bioassay test results.

With regard to the effects of toxicants on animal weight and growth it has been found that restricted feeding, enriching the diet in protein, altering nutrition, etc. has important influences on the expression of toxic phenomena by animals. The National Academy of Sciences has made recommendations concerning the nutrient requirements of common laboratory animals (i.e., mouse, rat, hamster, guinea pig, cat and dog) (SLAN, 1972). Often commercial feeds will provide a considerable excess of these nutrients so as to diminish the effects of degradative loss or altered bioavailability. Some minerals and vitamins, however, may be toxic when given in excess or may act to synergize the effects of the test agent. Thus, it is not really possible to make generalizations concerning exact levels of nutrients required in each bioassay. In chronic and subacute bioassays, however, it is crucial that an accurate specification of the animals' nutrient requirements not only be made but checked periodically by chemical analysis. This is especially true if the test compound is to be mixed in the feed and an ongoing program of measuring and recording the amount of feed (and concomitant test agent) must be performed. Homogeneous distribution of nutrients and test agents is, therefore, of ultimate importance in chronic and subacute studies.

3.5.6 Reproduction and Teratology Studies

Methods used to presently estimate reproductive and teratogenic hazards involve:

- Treatment span encompassing all or most of the period of organogenesis
- Use of rodent-rabbit species largely because of convenience in handling and low cost
- Multi-generation testing in order to screen in several ways for toxic effects at specific points in the mammal's reproductive cycle

A laboratory animal should be chosen by evidence that it metabolizes and distributes a test substance, transfers the substance across the placental barrier, and biotransforms the substance in utero in nearly the same manner as humans. Since a priori knowledge of the reproductive and teratologic effects of new test substances in humans may not be available, mammalian models must be chosen which approximate, to the best of our knowlege, human physiology, drug and toxicant metabolism, reproduction and embryology. A "3-generation bioassay" was suggested by the FDA in the mid-1960's in order to test for teratogenic effects resulting from low-dosage , long-term exposure to chemicals (e.g., food additives, pesticides, drugs, contaminants, etc.) in food. Animals, usually rats, are treated continuously through 3 reproductive cycles so as to provide opportunity for evaluating a multitude of parameters measuring reproductive performance, embryonic development, fetal and neonatal survivability in several successive generations. Although this is at present the best general screening procedure for judging test agents within a chronic, low-dosage bioassay, Wilson (1975) is of the opinion that more specialized tests are necessary for scrutinizing teratogenic and mutagenic potentials. Teratogenic short-term risks, for example, in the period of highest susceptibility (i.e., fetal organogenesis stage) may be masked due to maternal homeostatic dispersal and early induction of maternal hepatic enzymes which may protect the fetus in later stages. Wilson lists five separate tests for assaying developmental abnormalities that depend upon the duration of treatment:

- Testing throughout entire reproductive cycle -- whole generations
- Testing throughout pregnancy -- conception to term
- Testing throughout organogenesis -- primitive node to palatal closure
- Short-term (3 to 4 days) testing sequences during organogenesis
- Testing aimed at specific parameters -- mutagenesis, postnatal toxicity

The FDA guidelines of 1966 specified the following characteristics for 3 types of studies:

Type I - Fertility and general reproductive study

- Males given MTD (maximum tolerated dose) for 60 days before mating
- Females given MTD for 2 weeks before and during mating, pregnancy and lactation
- Young examined at 13 days gestation, term, and nursing

Type II - Teratology study

- Pregnant females treated days 6 through 15
- Young examined 1 to 2 days before term

Type III - Perinatal and postnatal study

- Dam treated last third of pregnancy and throughout lactation
- Young evaluated for survival and growth

The exact timing of maternal conception and implantation is very crucial in a well designed teratology study. When this knowledge of the maternal status is sketchy, the results may be misleading or highly variable due to a number of interferences or missed critical parameters which are listed in Table 3.5.3.

In an effort to circumvent these difficulties. Wilson (1975) proposed a number of new assay designs for improving current teratogenicity testing techniques. These included: (a) the use of some short-duration dosages, besides dosing throughout organogenesis, in order to avert enzyme induction and other adaptive responses on the part of the mother, (b) running tests in

TABLE 3.5.3 WAYS IN WHICH REPEATED TREATMENT PRIOR TO THE PEAK SUSCEPTIBLE PERIOD OF THE EMBRYO MAY PRODUCE MISLEADING RESULTS (Wilson, 1975)

Time of treatment	Primary effect	Secondary effect capable of altering test results
Before implantation	interference with implantation	no issue
Early organogenesis	early embryonic death	no issue
Before peak susceptibility	induction of catabolizing enzymes	reduced blood level during susceptible period
Before peak susceptibility	inhibition of catabolizing enzymes	increased blood level during susceptible period
Before peak susceptibility	liver pathology or reduced function	increased blood level during susceptible period
Before peak susceptibility	kidney pathology or reduced function	increased blood level during susceptible period
Before peak susceptibility	saturation of protein binding sites	increased blood level during susceptible period

species that more closely approximate human biotransformation mechanisms than do the rodent-rabbit species, (c) determining the embryo-toxicity threshold levels in animals so as to permit extrapolation downward to acceptable levels for humans, (d) expanding and improving postnatal function evaluations (e.g., sensory modalities, muscular coordination, and learning capabilities), and (e) reserving primates for agents needed, likely to be used, or of especially significant risk during human pregnancy.

3.5.6.1 Parental Observations--

In designing an experiment or bioassay to test the potential embryo-toxic or teratogenic effects of a substance, it is most desirable to effectively control the following variables to which the test parents are to be exposed:

- Quality of feed, bedding and drinking water
- Temperature, humidity, barometric pressure, amount and periodicity of light and noise
- Cage size, material, type of racks
- Group size unless animals are caged individually
- State of health and standard of laboratory care
- Species and strain of test animals
- Proof of male fertility, and fecundity
- Parity and time of mating of test females
- Accuracy of female exposure times to test substance and date of first insemination
- Treatment of concurrent control animals in all respects equal except for exposure to test substance

Teratogenicity tests in particular require meticulous record keeping throughout the assay period, careful necropsy of any female dying spontaneously and reporting of all dead and resorbed conceptuses. A pertinent report (U.S. EPA, 1977) stresses the importance of: (a) Running contemporaneous control animals chosen at random from the initial population of assay animals and coded along with the test animals in a manner that is "blind" to all except the principle investigators, (b) having a knowledge of the degree of spontaneous malformations and the range of variants (skeletal and visceral) characteristic of the chosen strain of test animal, and (c) availability or morphologic atlases of this chosen strain by which accurate comparisons may be made of the type and degree of malformation induction. Although the FDA required only 2 dosage levels be tested, Wilson and others have recommended the testing of a broader range of levels such as: 0.5, 0.25, and 0.125 times the acutely toxic, maternal LD50 dosage. The degree of variability and level of spontaneous malformation, which is evident in all species to some extent, further supports the contention that not just rats and mice, but also dogs, cats, pigs, sheep and other mammals ought to be assayed since their placental arrangement is more akin to human than is that of rodents and rabbits. Since a single ideal animal does not exist which would satisfy all these criteria, Wilson has suggested successive levels of evaluation in different animals as depicted in Table 3.5.4:

TABLE 3.5.4 A NEW CONCEPT IN TERATOGENICITY TESTING BASED ON MULTILEVEL TESTS IN DIFFERENT TYPES OF ANIMALS (Wilson, 1975)

Order of test	Purpose	Suitable species	No. of pregnant animals
First level	find embryotoxic dose range	rat, mouse, hamster, or rabbit	130–150
Second level	confirm or adjust above	a carnivore or an ungulate	40–60
Third level	only if second level results are equivocal	alternate to that used in second level	40–60
Fourth level	only if use in human pregnancy needed or likely	macaque monkey or baboon	40–50

a. Tests would terminate at second or third level in most instances.
b. Whenever possible selection should be made on the basis of metabolic similarity to man.

Large numbers of inexpensive animals, such as rats or mice, could be first used for finding the general embryotoxic dosage range. The second-level tests would utilize large animals (either carnivore or ungulate) whose reproductive anatomy and physiology is more like that of humans, and upon whose results a more accurate estimate of potential human teratogenicity might be based. A third level of testing in another subprimate mammal might be necessary only if the results of the first-and second level bioassays remain questionable. Fourth-level tests involving nonhuman primates have to be restricted to substances which pregnant women are inadvertently exposed to in significantly large quantities and for which an epidemiologic suspicion has been deduced, and (b) medicants and drugs which are necessary for control of disease or severe discomfort during pregnancy. Extrapolation of these results to the human condition, however, will always be difficult even with retrospective epidemiologic surveillance of human populations.

3.5.6.2 Fetal Manifestations--

Several uncertainties exist in the suggestion of any inferences between the results in animal models and the expected effects in human infants due to developmental differences between species. An important consideration made (U.S. EPA, 1977) is that of the time course of intrauterine development, irrespective of life span or gestation period. In this review of teratogenicity screening assays, it is pointed out that the preimplantation period (during which cells are undifferentiated and hence not yet demonstrating teratogenic responses) varies from 4.5 days in hamsters to 6.15 days in man to 10 days in sheep. Organogenesis takes place in the first 6 weeks in human development, while a proportionally longer or shorter period out of the total gestation time is evident in other mammals. The chronology of structural developments in the central nervous system (CNS), a prime target for teratogenesis, when measured histologically varies greatly among species with the final steps for humans occurring much later during the postnatal period. Other major differences between the fetal development of humans versus that of other mammals as documented are:

- Single implantation in humans versus multiple implantations in many test animals which can cause variations in the proportion of embryos and fetuses resorbed, in number of abortions, still births, etc.
- Differences in endocrinology, metabolism, pharmacology, pharmacokinetics and nutrition
- Inbred nature and genetic characteristics of laboratory animals as compared with the more randomly bred and larger, more chronically exposed human populations.

Another potential fetal variability factor causing dosage fluctuations and resulting from maternal homeostatic adaptions may be avoided by testing only short-duration dosages. Fewer animals are needed in each short-term assay group than is the case when the dosages are given throughout organogenesis. Later, it is recommended that the highest dosage that produces no increase in embryotoxicity during the short-term treatment spans should be given to another test group for assay over full period of organogenesis. According to Wilson, the few animals that are required in these separate short-duration assays still provide more precise information than those from a larger-scale single experiment.

Demonstration of a level of test substance which is embryotoxic independent of levels which are teratogenic has received little attention and needs to have more emphasis in the future. A threshold of teratogenic potential for a particular toxicant, in fact, may be substantially close to the embryotoxic level, in which case the chemical may still be used as long as concentrations do not exceed these threshold limits. If applied in sufficient dosage at a susceptible developmental stage of a laboratory mammal, a toxicant may prove to be embryotoxic, while higher doses at later or earlier periods would be needed to demonstrate a teratogenic effect. This information should be required before setting safe tolerance limits.

Postnatal functional capabilities must receive greater emphasis in the future due to the fact that: (a) nervous and endocrine systems or organs are not fully functional at birth in humans and other species, and (b) functional deficiencies in these systems or organs may not readily be reflected in poor growth and/or survivability postnatally. Thus, it is crucial that postnatal functional evaluations be extended beyond weaning and be included in growth and survival records. Specific tests of sensory modalities, muscular coordination, learning capabilities need to be refined so as to allow for improved quantitation and concomitant quality control specification.

3.5.6.3 Mathematical and Statistical Analyses--

As a result of these differences in fetal responsiveness between species, there is a lack of consensus as to the best experimental design or approach. Although it would be deemed ideal, a program of single dosages to test each specific organ and tissue sensitivity at the time of the most rapid proliferation would be far too costly. Multiple doses, however, have been reported to yield cumulative effects and/or few malformations due to development of metabolic tolerance by the parent female. So-called "equivalent dosages" (i.e., dosages weighted for known tissue sensitivities) have been advocated as a result. The notion of a "threshold" concept of dosage-responsiveness has been recommended, as well as the concept of a "teratogenic ratio" (the ratio of the maternally-measured toxicity of a chemical to the embryotoxic level of this chemical) has been proposed as the best predictor of teratogenic potential (Robson, 1970). Thus, there appear to be several types of mathematical analyses which have achieved varying degrees of acceptance by the scientific community, no one of which provides answers to all questions.

Collins and Collins (1976) recommend that a table of random numbers be used in order to assign animals to control or experimental groups, ensuring that each pregnant female is treated as an independent sampling unit. Percentages of affected fetuses per litter and incidence of affected fetuses per total number of fetuses should be reported. The best experimental design is one in which the evaluators are "blind" as to which dams are of the test group and which are of the control through a labeling code known only to the principal investigators. At each dosage level quantitation must be made of each specific anomaly and of the total anomalies that appear in each grouping, since the sum of all anomalies considered together in a specific grouping may indicate a teratogenic effect. A series of at least three dosage levels, allows for preliminary dose-response correlations. It should later be required that both the embryotoxic level and the no-observed-effect level be determined, as both are valuable in the regulation of potentially toxic substances. Safe human exposure levels should be a fraction (e.g., 1/1000, 1/100, 1/20) of the calculated embryotoxic dosage. Although absolute certainty is impossible to achieve, the estimated risk of teratogenic effects from new substances or chemical combinations must be assessed if we are to avoid subjecting our offspring to unknown hazards.

3.5.7 Mammalian Mutagenicity Tests

3.5.3.1 Dominant Lethal Bioassay--

Gross genetic damage has been induced by x-rays and chemical mutagens and has been measured by a technique termed the dominant lethal test. The association of zygote lethality with dominant chromosomal aberrations, such as chromosomal translocations that result in nonviable zygotes, has been used as evidence in the correlation of chemically-induced damage with heritable mutation(s), which in this case are lethal. In their recommendations of the general practice of dominant lethal assays, Green et al. (1976) suggested the following calculations and measurements be utilized "to evaluate statistically the results of dominant lethal studies":

- Fertility Index - This index is used to analyze the number of pregnant females per number of mated females via the chi-square comparison of the values for each treatment group versus the calculated control values. A trend for linearity of proportions may be used to ascertain whether this index is linearly related to arithmetic or logarithmic dose of test agent.
- Total Number of Implantations Index - Significant differences between average numbers of implantations per pregnant female in each treatment group against control values are assayed statistically via the t-test. Linear regression analysis is used to determine if this index is related to arithmetic or logarithmic dose.
- Total Number of Corpora Lutea - Again, significant differences are ascertained by t-test comparison between control and treatment values, and linearity by regression analysis.
- Preimplantation Losses Count - These losses are measured by direct counting of the number of corpora lutea. The preimplantation losses for each female are mathematically transformed to the Freeman-Tukey arc-sine values and the t-test is then used to compare each treatment value with those controls.
- Dead Implantation Count - These counts are statistically analyzed in the same manner as preimplantation counts.
- Proportion of Females with One or More Dead Implants - The chi-square test is suggested for comparing control and treatment values, while the trend for linear proportions may be used to determine if these proportions are linearly related to arithmetic or logarithmic dose. Alternatively, probit regression analysis may be employed to test for these types of linear correlation.
- Proportion of Females with Two or More Dead Implants - Mathematic analyses of these proportions are carried out in the same manner as that for the previous proportions.
- Dead Implantations per Total Implantations - Using the count data from each female, a Freeman-Tukey arc-sine transformation is obtained for each control and treatment value. Comparison of these values is then made via the t-test.

- Variation between Males with Time - Using a nested model, the analysis of variance between individual males and between results obtained in individual test weeks or pairs of test weeks.

Although the dominant lethal bioassay is thought to reflect chromosomal aberrations, it cannot by definition measure heritable chromosomal effects other than those that produce lethality. Many agents which induce dominant lethality, however, are known to cause heritable chromosomal aberrations when investigated by non-mammalian or in vitro bioassays. The actual measure in the dominant lethal test is early fetal loss (Green et al., 1977; Embree et al., 1977 ; Epstein et al., 1972). The working hypothesis of this test is that abnormalities produced in sperm may lead to developmental errors causing early death of a zygote.

There is some scientific concern that dominant lethality as measured by fetal death or wastage may occur for reasons other than chromosomal mutation. As a result, problems may result in the interpretation of the observations made in using this system in a definitive manner. The utility of the dominant lethal assay, however, stems from its ease of performance and the positive correlation that has been established between it and other animal systems.

EXAMPLE: DOMINANT LETHAL BIOASSAY

Experimental Design

- Dosing and mating schedules have generally been designed so as to permit a sampling of potential effects on all sperm cell stages through meiosis. A shortened approach to this bioassay has also been presented (Green et al., 1977) which is outlined below along with suggested quality control steps.

Design Outline

- Adult male rodents treated for 5 consecutive days with acute or subacute dosage; each is then mated to 2 virgin females each week for 8 to 10 week periods; each female is sacrificed 14 days from the midweek of co-housing; inclusion of untreated males separately tested as negative, vehicles control animals and a second group of males dosed via the same route with known mutagen as positive control animals.
  QUALITY CONTROL -- Same as that outlined in acute toxicity bioassays (Section 3.5.4.1). Especially important are age, weight, good health and proven fertility checks of males prior to bioassay. If known, the spontaneous frequency of dead or abnormal implantations should be specified for each strain and species tested.

Bioassay and Statistical Analyses

- The number of corpora lutea, as well as the count of the dead implantations per pregnant female is transformed by Freeman-Tukey square rooting and subsequently subjected to t-test analysis; the count of females with 1 or more dead implantations and the number of females with 2 or more dead implantations are subjected to chi-square analysis.
  QUALITY CONTROL -- Same as that outlined in acute toxicity bioassays

(section 3.5.4.1) particularly with respect to record keeping, "blind status" of examining pathologist, and substantial results with positive control animals.

Results obtained in a test of triethylenemelamine (TEM) showed that this shortened approach reduced the amount of data usually required with no loss of pertinent information concerning inherited lethality. Information concerning what stage spermatogenesis is adversely affected, however, requires the more elongated bioassy.

3.5.7.2 In Vivo Cytogenetics and Cytotoxicity Bioassays--

Cytologic and cytogenetic bioassays utilizing the techniques of visible light and transmission electron microscopy have been available for detecting chromosomal anomalies in mammals exposed to potential toxicants in vivo. These procedures fall into two categories, those that detect damage (transmittable and non-transmittable) expressed in germ cells during early embryogenesis, and those whereby somatic tissue cells are assessed for nuclear, chromosomal, cytoplasmic and mitochondrial damage within cells of critical tissues and organs. The somatic tissue used is usually bone marrow (Legator et al., 1969; Georgian, 1975; Majumdar et al., 1976), while testes have been assayed in germinal cell studies. Human lymphocytes may also be tested if accidental or chronic use exposure has occurred and information is available on the cytomorphology of the lymphocytes prior to exposure (Lubs and Samuelson, 1967).

Regarding quality control aspects, Cohen and Hirschhorn (1971) have stressed that replicates of each cytologic sample in the form of multiple slides be examined by cytopathologists in a "blind" manner (i.e., coded) on different days in randomized sequences. To overcome any potential observer biases, these slides should, if possible, be further examined and scored by 2 other microscopists, and their scores averaged. It was also suggested that all repeated experiments be statistically tested for "homogeneity of variances," while tests of significance between results of treated and untreated groups be based on statistically significant differences in the mean scores and variances thereof via t-test and F-test techniques, respectively. A summary of these and other critical points is here presented in outline form:

Summary of Experimental Design Outline

- Acute Studies - 3 groups of 5 male albino rats are used for each agent at each dosage level; and equal number of rats (negative controls) treated with vehicle only, a third group (positive controls) dosed with triethylenemelamine (TEM); test agent(s) orally administered at one of 3 dosage levels (LD5, LD25 or LD50, and "usage level"); 2 to 4 hours prior to scheduled sacrifice, 4 mg/kg of colcemid is injected intraperitoneally; of the 3 groups at each dosage level, one is sacrificed at 6 hours, one at 24 hours, and the last at 48 hours after treatment.
  QUALITY CONTROL -- Seek advice of biostatistician on group size and methods of statistical analysis to be used in scrutinizing data. Additionally, the quality control steps outlined for acute toxicity

bioassays (Section 3.5.4.1) should be performed.

- Subacute Studies - 3 groups of 5 males are used for each test agent with negative controls as in the acute studies, but here the oral dosage is applied for 5 days at 24 hour intervals at the same 3 levels (LD5, LD25 or LD50, and "usage level"). The assays are terminated as described above for acute studies.
  QUALITY CONTROL -- Same as above.

- Observations and Bioassays -- Bone marrow cells are taken from the femurs of each rat and prepared in a routine manner; the percentage of single and multiple metaphase chromosomal aberrations (i.e., frequency of chromatid breaks, fragmentations, chromosomal interchanges and ring formations) as well as incidence of swollen mitochondria, increased cytoplasmic granulation, vacuolizations, growth of abnormally large cells, and/or cellular membrane damage is recorded.
  QUALITY CONTROL -- All scoring of bone marrow cells is performed on coded slides. In addition to the steps outlined for acute toxicity bioassays (Section 3.5.4.1), the following data should be recorded: (a) Vernier and magnification settings used in the microscopic observations, (b) written definitions of each chromosomal aberration accompanied by an example photo, and (c) identification of chromosomal gaps separately.

3.5.7.3 Specific Locus Bioassay for Detecting Gene Mutations--

The mouse-specific locus test has been used for the detection of specific gene mutations that have been induced in the germ cells of rodents (generally mice) exposed to chemical or physical (x-rays) mutagens (Russell, 1951; Searle, 1975; Cattanach, 1971). The test requires stocks of at least 2 strains of mice, one that is homozygous for dominant wild-type alleles at 7 loci and one that is homozygous recessive at these same loci. Mice of the wild-type stock are treated with the presumptive mutagen, and after a specific duration, allowing for metabolism and induction of mutational events in the males' germ cells, the treated males are mated with mice of the homozygous recessive tester strain. The chosen specific duration between treatment and mating determines the stage of the developmental germ cells which are assayed. Induced mutations at these loci can be scored as changes in coat color, eye color, ear size in 1 week old progeny, etc. Dominant mutations at other loci may also be observed in these litters if they are of a visible type.

A few disadvantages of this bioassay are that it requires large numbers of animals in order to obtain marginal sensitivity, and laboratories with the required mouse colonies and expertise are limited. Moreover, it is of concern that not all types of mutagenic events (e.g., base-pair substitutions) can be readily detected by simply phenotypic observation. Future electrophoretic typing of sera proteins may help in detecting these other events. The bioassay at present, however, has the great advantage of estimating germ cell risks to genes in response to acute and subacute dosages in an intact animal via routes analogous to the possible pathways of human exposure. Critical points pertaining to these problems are summarized below:

Summary of Design Outline

- Mice of the inbred strains C3H and 101 and of the multiple recessive T-stock, each of 7 to 8 weeks of age are required. As outlined by Searle (1975), 200 C3H females per test or control group should be mated with 100 male 101 mice in trios, and the 3H1 male offspring mated with 200 T-stock females in a scheduled manner to yield at least 50,000 progeny at each of 2 C3H-parental dosage levels, as well as for controls.
  QUALITY CONTROL -- Seek advice of biostatistician on group sizes. Additionally the quality control steps outlined for acute toxicity bioassays (subsection 3.5.4.1) should be performed. To detect a true doubling above the natural mutation frequency, Searle (1975) estimated that at least 30,000 classifiable offspring (preferably more) be examined.

Bioassay and Statistical Analyses

- All young from the mating of 3H1 males with T-stock females should be counted, sexed and carefully examined with respect to the 4 early phenotypes, and after 18 to 19 days, 10 final phenotypes. The calculated mutation for each parental dosage or control group per locus is given by total number of specific locus mutations divided by the product of the number of young classified times the number of loci. Statistical comparison of control and test results may be performed by Fisher's "exact treatment" method from a 2 x 2 table as described by Searle (1975). A chi-square test may also be used, but Yates correction for continuity should be applied.
  QUALITY CONTROL -- The "blind" status of the examiners, the care in recording all examinations and the confirmation all genetic loci tests against example mouse photos are key quality control steps.

The specific locus bioassay can provide direct information relevant to assessing human genetic risks. Unfortunately, large numbers of offspring must be examined since this bioassay: (a) Detects only 6 or 7 recessive loci out of many thousands, (b) assays only forward mutations at these loci which yield visible phenotypic changes, (c) permits determination of the nature of these mutations only with difficulty unless they involve both the *d* and *se* loci, (d) may allow non-mutational events to be scored as mutations, and (e) may miss counting heterozygous mutatants that do not survive long enough to be recognized. It is likely that future research, however, will improve the number of loci that can be assayed and will allow further reductions in the detectability limitation of this bioassay technique.

3.5.8 References

AOAC. 1975. Official Methods of Analysis, 12th Edition. Association of Official Analytical Chemists, Washington, D.C.

Barnes, J. M., and D. F. Heath. 1964. Some toxic effects of dieldrin in rats. Brit. J. Ind. Med. 21: 280-282.

Boyd, C. E., and E. M. Boyd. 1962. The chronic toxicity of atropine administered intramuscularly to rabbits. Toxicol. Appl. Pharmacol. 4: 457-467.

Boyd, E. M. 1968. Predictive drug toxicity. Assessment of drug safety before human use. Can Med. Ass. J. 98: 278-293.

Case, M. T., J. K. Smith, and R. A. Nelson. 1976. Chronic oral toxicity studies of nefopam hydrochloride in rats and dogs. Toxicol. Appl. Pharmacol. 36: 301-306.

Cattanach, B. M. 1971. Specific locus mutations in mice. In: Chemical Mutagens: Principles and Methods for Their Detection, Vol. 2. A. Hollaender (ed.). Plenum Press, New York, N.Y. pp. 535-539.

Cohen, M. M., and K. Hirschhorn. 1971. Cytogenetic studies in animals. In: Chemical Mutagens: Principles and Methods for Their Detection, Vol. 2. A. Hollaender (ed.). Plenum Press, New York, N.Y. pp. 515-534.

Collins, T. F. X., and E. V. Collins. 1976. Current methodology in teratology research, Chapter 6. In: Advances in Modern Toxicology, Vol. 1, Part 1, New Concepts in Safety Evaluation. M. A. Mehlman, R. E. Shapiro, and H. Blumenthal (eds.). John Wiley and Sons, New York, N.Y.

Embree, J. W., J. P. Lyon, and C. H. Hine. 1977. The mutagenic potential of ethylene oxide using the dominant-lethal assay in rats. Toxicol. Appl. Pharmacol. 40: 261-267.

Epstein, S. S., E. Arnold, J. Andrea, W. Bass, and Y. Bishop. 1972. Detection of chemical mutagens by the dominant-lethal assay in the mouse. Toxicol. Appl. Pharmacol. 23: 288-325.

Georgian, L. 1975. The comparative cytogenetic effects of aldrin and phosphamidon. Mutat. Res. 31: 103-108.

Goldstein, A. 1964. Biostatistics - An Introductory Test, Chapter 1, p. 1-33. Macmillan Publ. Co., New York, N.Y.

Goodman, L. S., and A. Gilman (eds.). 1975. The Pharmacologic Basis of Therapeutics, Chapter 1-General Principles, by E. Fingl and D. M. Woodbury, pp. 1-46.

Gove, P. B. (chief ed.). 1976. Webster's Third New International Dictionary of the English Language, Unabridged. G. and C. Merriam Co., Publ., Springfield, Mass. p. 581.

Green, S., E. Zeiger, K. A. Palmer, J. A. Springer, and M. S. Legator. 1976. Protocols for the dominant lethal test, host-mediated assay and in vivo cytogenetic test used in the FDA's review of substances in the GRAS List. J. Toxicol. Environ. Health 1: 921-928.

Green, S., F. M. Moreland, and W. G. Flamm. 1977. A new approach to dominant-lethal testing. Toxicol. Appl. Pharmacol. 39: 549-552.

Grice, H. C., and T. Da Silva. 1973. The Testing of Chemicals for Carcinogenicity, Mutagenicity and Teratogenicity. Ministry of Health and Welfare of Canada, Ottawa, Canada. Section on Chemical Carcinogenesis Testing, p. 34-49.

Hayes, W. J. 1975. Toxicology of Pesticides. Chapter 2 - General principles: dosage and other factors influencing toxicity, p. 37-106. Williams and Wilkins, Baltimore, Md.

Hinkle, D. K. 1977. Personal communication addressing quality assurance and control in animal laboratories. U.S. Environmental Protection Agency, Research Triangle Park, N.C. February 4, 1977.

ILAR. 1974a. Guide for the Care and Use of Laboratory Animals. Institute for Laboratory Animal Resources, DHEW Publication No. NIH 74-23. Public Health Service, National Institutes of Health, Bethesda, Md.

Ibid. 1960. Standards for the Breeding, Care and Management of Syrian Hamsters. NAS-NRC, Bethesda, Md.

Ibid. 1964. Standards for the Breeding, Care, and Management of Guinea Pigs. NAS-NRC, Bethesda, Md.

Ibid. 1964. Standards for the Breeding, Care and Management of Laboratory Cats. NAS-NRC, Bethesda, Md.

Ibid. 1967. Standards for the Breeding, Care and Management of Laboratory Rabbits. NAS-NRC, Bethesda, Md.

Ibid. 1969. Procurement Specification (Contract Clause), VI. Colony-Produced Cats. NAS-NCR, Bethesda, Md.

Ibid. 1969. Procurement Specification (Contract Clause), V. Kennel-Produced Dogs. NAS-NRC, Bethesda, Md.

Ibid. 1971. A Guide to the Infectious Diseases of Mice and Rats. NAS-NRC, Bethesda, Md.

Ibid. 1973. Procurement Specification (Contract Clause) IX. Defined Laboratory Rodents and Rabbits. NAS-NRC, Bethesda, Md.

Ibid. 1974b. A Guide to the Infectious Diseases of Guinea Pigs, Gerbils, Hamsters and Rabbits. NAS-NRC, Bethesda, Md.

Ibid. 1976. Long-Term Holding of Laboratory Rodents. ILAR News, 19(4); L1-25. NAS-NRC, Bethesda, Md.

Ibid. 1977. Laboratory Animal Management: Rodents. ILAR News, 20(3): L1-15. NAS-NRC, Bethesda, Md.

Legator, M. S., K. A. Palmer, S. Green, and K. W. Petersen. 1969. Cytogenetic studies in rats of cyclohexylamine, a metabolite of cyclamate. Science 165: 1139-1140.

Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96: 99-113.

Loomis, T. A. 1974. Essentials of Toxicology. Chapter 13 - Toxicologic testing methods, p. 177-215. Lea and Febiger, Publ., Philadelphia, Pa.

Lubs, H. A., and J. Samuelson. 1967. Chromosome abnormalities in lymphocytes from normal human subjects. Cytogenetics 6: 402-411.

Majumdar, S. K., H. A. Kopelman, and M. J. Schnitman. 1976. Dieldrin-induced chromosome damage in mouse bone-marrow and WI-38 human lung cells. J. Hered. 67: 303-307.

McLean, A. E. M., and E. K. McLean. 1969. Diet and toxicity. Brit. Med. Bull. 25: 278-281.

NCI. 1976. National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. National Cancer Institute, DHEW Publication No. NIH 76-900.

Rand, M. C., A. E. Greenberg, and M. J. Taras (eds.). 1975. Standard Methods for the Examination of Water and Wastewater. 14th Edition. American Public Health Association, American Water Works Association, and Water Pollution Control Federation. Washington, D.C.

Robson, J. M. 1970. Testing drugs for teratogenicity and their effect on fertility. Brit. Med. Bull. 26: 212-216.

Russell, W. L. 1951. X-ray induced mutations in mice. Cold Spring Harbor Symp. Quant. Biol. 16: 327-336.

Searle, A. 1975. The mouse-specific locus test. Mutat. Res. 31: 298-325.

Slan. 1972. Nutrient Requirements of Laboratory Animals. National Academy of Sciences, National Research Council, Subcommittee on Laboratory Animal Nutrition, Washington, D.C.

Sokal, R. R., and F. J. Rohlf. 1969. Biometry, Chapter 7 - Estimation of

Hypothesis Testing, p. 127-174. W. H. Freeman and Co., San Francisco, Calif.

Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U.S. Department of Health, Education and Welfare, NIH 76-801. pp. 11-29, 50.

Steinberg, M., W. A. Akers, M. Weeks, A. H. McCreesh, and H. I. Maibach. 1975. A comparison of test techniques based on rabbit and human skin responses to irritants with recommendations regarding the evaluation of mildly or moderately irritating compounds. Chapter 1 in: Animal Models in Dermatology. H. Maibach (ed.). P. 1-11. Churchill Livingstone, Publ., New York, N.Y.

U.S. EPA. 1977. Survey and Evaluation of Techniques Used in Testing Chemical Substances for Teratogenic Effects. U.S. Environmental Protection Agency, Office of Toxic Substances, EPA-560/5-77-007.

U.S. FDA. 1959. Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics. U.S. Food and Drug Administration, Division of Pharmacology, Washington, D.C.

U.S. FDA. 1966. Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use. U.S. Food and Drug Administration, Washington, D.C.

U.S. Pharmacopeia. 1975. XIX Edition, pp. 581-794. U.S.Pharmacopoeial Convention, Inc., Rockville, Md.

Weil, C. S., and D. D. McCollister. 1963. Relationship between short- and long-term feeding studies designing an effective toxicity test. J. Agr. Food Chem. 11: 486-491.

Weisburger, E. K. 1975. Critical evaluation of the methods used for determining carcinogenicity. J. Clin. Pharm. 15: 5-15.

Weisburger, J. H. 1976. Bioassays and tests for chemical carcinogens, Chapter 1. In: Chemical Carcinogens, ACS Monograph No. 173, pp. 1-23. American Chemical Society, Washington, D.C.

Wilson, J. G. 1975. Reproduction and teratogenesis: current methods and suggested improvements. J. Ass. Offic. Anal. Chem. 58: 657-667.

Zbinden, G. 1973. Progress in Toxicology. Chapter 2 - Formal toxicology, pp. 4-27. Springer-Verlag, New York, N.Y.

APPENDIX A

CHECK LIST FOR PLANNING TEST PROGRAMS

The following check list is taken from: Bicking, C. A., 1954. Some uses of statistics in the planning of experiments, Industrial Quality Control 10: 20-23.

CHECK LIST FOR PLANNING TEST PROGRAMS

A. *Obtain a Clear Statement of the Problem*
  1. Identify the new and important problem area
  2. Outline the specific problem within current limitations
  3. Define exact scope of the test program
  4. Determine relationship of the particular problem to the whole research or development program

B. *Collect Available Background Information*
  1. Investigate all available sources of information
  2. Tabulate data pertinent to planning new program

C. *Design the Test Program*
  1. Hold a conference of all parties concerned
     a. State the propositions to be proved
     b. Agree on magnitude of differences considered worthwhile
     c. Outline the possible alternative outcomes
     d. Choose the factors to be studied
     e. Determine the practical range of these factors and the specific levels at which tests will be made
     f. Choose the end measurements which are to be made
     g. Consider the effect of sampling variability and of precision of test methods
     h. Consider possible inter-relationships (or "interactions") of the factors
     j. Determine limitations of time, cost, materials, manpower, instrumentation and other facilities and of extraneous conditions, such as weather
     k. Consider human relations angles of the program
  2. Design the program in preliminary form
     a. Prepare a systematic and inclusive schedule
     b. Provide for step-wise performance or adaptation of schedule if necessary
     c. Eliminate effect of variables not under study by controlling, balancing, or randomizing them
     d. Minimize the number of experimental runs
     e. Choose the method of statistical analysis
     f. Arrange for orderly accumulation of data
  3. Review the design with all concerned
     a. Adjust the program in line with comments
     b. Spell out the steps to be followed in unmistakable terms

D. *Plan and Carry Out the Experimental Work*
   1. Develop methods, materials, and equipment
   2. Apply the methods or techniques
   3. Attend to and check details; modify methods if necessary
   4. Record any modifications of program design
   5. Take precautions in collection of data
   6. Record progress of the program

E. *Analyze the Data*
   1. Reduce recorded data, if necessary, to numerical form
   2. Apply proper mathematical statistical techniques

F. *Interpret the Results*
   1. Consider all the observed data
   2. Confine conclusions to strict deductions from the evidence at hand
   3. Test questions suggested by the data by independent experiments
   4. Arrive at conclusions as to the technical meaning of results as well as their statistical significance
   5. Point out implications of the findings for application and for further work
   6. Account for any limitations imposed by the methods used
   7. State results in terms of verifiable probabilities

G. *Prepare the Report*
   1. Describe work clearly giving background, pertinence of the problems and meaning of results
   2. Use tabular and graphic methods of presenting data in good form for future use
   3. Supply sufficient information to permit reader to verify results and draw his own conclusions
   4. Limit conclusions to objective summary of evidence so that the work recommends itself for prompt consideration and decisive action

APPENDIX B.

GOOD ANIMAL CARE LABORATORY PRACTICES

Designation	Subject	Page
CBO-1	Laboratory Animal Care Personnel	B-2
CBO-2	Provisions for Emergency Laboratory Care	B-5
CBO-3	Carcinogen Bioassay Pathology Personnel	B-6
CBO-4	Safety of Animal Care Personnel	B-8
CBO-5	Preparation for Shipment and Transportation of Laboratory Animals	B-12
CBO-6	Receipt and Quarantine of Laboratory Animals	B-15
CBO-7	Weighing of Laboratory Animals	B-18
CBO-8	Examination of Laboratory Animals for General Health	B-19
CBO-9	Examination of Rodents for Parasites	B-21
CBO-10	Randomization, Assignment, and Identification of Animals	B-22
CBO-11	Storage of Feed, Bedding, and Equipment for Laboratory Animals	B-26
CBO-12	Dose Preparation and Analysis for Chemicals to be Administered by Procedures other than Inhalation	B-29
CBO-13	Feeding of Laboratory Animals	B-32
CBO-14	Generation and Analysis of Test Atmosphere of Chemicals Evaluated by the Inhalation Method Program	B-34
CBO-15	Watering of Laboratory Animals	B-37
CBO-16	Changing of Litter or Bedding, Changing of Laboratory Animal Cages, and Disposal of Waste	B-40
CBO-17	Maintenance of Optimal Environmental Conditions for Laboratory Animals	B-44
CBO-18	Sanitation of Equipment and Supplies for Laboratory Animals	B-51
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CBO-20	Vermin Control in Animal Facilities	B-56
CBO-21	Sacrifice of Laboratory Animals (Euthanasia)	B-58
CBO-22	Disposal of Dead or Sacrificed Animals and Tissues	B-60
CBO-23	Disposal of Radioactive Wastes Associated with Laboratory Animal Experiments	B-62
CBO-24	Disposition of Carcinogen Bioassay Pathology Material	B-64
CBO-25	Required Information	B-67
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Carcinogen Bioassay Program　Specification No. CBO-1

Type: GOOD ANIMAL CARE LABORATORY PRACTICE				**Sheet** 1	**Of** 3
Subject: LABORATORY ANIMAL CARE PERSONNEL					
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers laboratory animal care personnel used in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 General

3.1.1 Animal care program shall be directed by veterinarians having specialized training or experience in laboratory animal medicine.

3.1.2 The employment of a full-time staff specifically concerned with the animal care program is recommended. The staff shall include the professional and supporting personnel necessary to implement the veterinary, animal husbandry, and administration aspects of the program.

3.2 Supervisors

3.2.1 All personnel and facilities for maintaining laboratory rodents shall be directly supervised by a professionally qualified person In addition, services of a veterinarian trained in a laboratory animal medicine should be available either on a permanent basis or as a part-time consultant.

3.3 Technicians

3.3.1 Technicians employed as caretakers of laboratory animals shall be trained in formal courses designed for that specific purpose or shall undergo extensive on-the-job training under close supervision.

3.3.2 Caretakers shall be certified in their job specialty by a nationally recognized certification board.

3.3.3 Caretakers shall be able to recognize symptoms of disease and other abnormalities.

Subject:	Date:	Sheet	Of
LABORATORY ANIMAL CARE PERSONNEL		2	3

3.4 Drivers

3.4.1 Each driver or assistant shall be qualified to handle and care for laboratory animals being transported and to provide needed services in emergencies. This qualification may consist of either completion of an approved animal care technician course or equivalent practical experience in animal care.

4. QUALITY CONTROL

4. 1. Animal technicians should be divided into 2 grades.

4. 1.1 Grade A (lower grade), minimum of 9 years of schooling, probationary period of at least 3 months; completion of a formal course of instruction; a minimum 2 year period of service before examination.

4. 1.2 Grade B (higher grade), similar requirements for schooling and probationary period; completion of an advanced formal course of instruction; a minimum three year service period as a Grade A technician before examination.

4. 2. The education of laboratory animal technicians should be based on the following outline:

4. 2.1 Introduction to animal care.

4. 2.2 Life, living matter and biological organization.

4. 2.3 Structure and function (skeletal and muscular system, integument; circulatory and respiratory systems; digestive and excretory systems; nervous system and sense organs; endocrine systems; reproductive system).

4. 2.4 Genetic and mating system.

4. 2.5 Nutrition and metabolism.

4. 2.6 Handling.

4. 2.7 Animal health and disease.

4. 2.8 Sanitation and hygiene.

4. 2.9 Housing and equipment design.

4. 2.10 Administration, management, record keeping.

4. 2.11 Shipping and receiving of animals.

4. 2.12 Safety

4. 2.13 Animal experimentation

4.3. Apart from scholastic achievements the animal technician should have a natural aptitude for dealing with animals and keen sense of discipline and responsibility thus ensuring that the requirements of the animal will always be uppermost in his mind (6.5, p. 116).

Subject: LABORATORY ANIMAL CARE PERSONNEL	**Date:**	**Sheet** 3	**Of** 3

5. PACKAGING

N/A

6. REFERENCE DOCUMENTS

6.1 Code of Federal Regulations, Title 9, Chapter 1, Subchapter A. Animal Welfare. Parts 1, 2 and 3, May, 1972.

6.2 Guide for the Care and Use of Laboratory Animals. 1974. U. S. Department of Health, Education and Welfare, NIH 74-23.

6.3 Procurement Specification VII. Rodents. 1969. Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D. C.

6.4 Rodents. 1969. Standards and Guidelines for the Breeding, Care and Management of Laboratory Animals. National Academy of Sciences, Washington, D. C.

6.5 The UFAW Handbook on the Care and Management of Laboratory Animals, 1972, 4th edition. UFAW Staff (eds.). Churchill Livingstone, Edinburgh and London.

Carcinogen Bioassay Program Specification No. CBO-2

Type: GOOD ANIMAL CARE LABORATORY PRACTICE				**Sheet** 1	**Of** 1
Subject: PROVISIONS FOR EMERGENCY LABORATORY CARE					
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers the steps taken to provide for emergency laboratory animal care.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Provision must be made for the emergency care of animals.

3.1.1 Animal care supervisors and personnel available for emergency duty shall be identified and alerted to the necessary emergency care procedures.

3.1.2 A list of the animal care supervisors and designated personnel shall be prominently posted at the laboratory's central telephone center and in the security department if one exists.

3.1.3 Laboratory security personnel and fire and police officials should know how to reach the individuals responsible for emergency care.

3.2 Emergency animal care personnel shall be called to duty promptly upon discovery of any emergency involving animals or records.

4. QUALITY CONTROL

Periodically update the name, telephone number and address of the responsible individuals. The objective is to assure that animals will be cared for should an emergency arise.

5. PACKAGING

N/A

6. REFERENCE DOCUMENTS

6.1 Guide for the Care and Use of Laboratory Animals. 1974. U. S. Department of Health, Education and Welfare, NIH 74-23.

Carcinogen Bioassay Program Specification No. CBO-3

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 2

Subject:

CARCINOGEN BIOASSAY PATHOLOGY PERSONNEL

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers personnel required for all pathology work with small rodents employed in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 A board-certified pathologist (veterinary or medical) experienced in laboratory animal pathology shall be responsible for all pathology procedures, evaluations, and reporting. Persons not board-certified may be acceptable if appropriate training and experience judged to be satisfactory by program management can be demonstrated.

3.2 Histology technician(s) shall be supervised by an HT/ASCP registered technician who is qualified as judged by program management. Persons not certified may be acceptable if they have had appropriate training and experience that is satisfactory in the judgement of program management.

3.3 Prosectors shall be trained and experienced in laboratory animal dissection and must be able to recognize and describe gross abnormalities. "Careful performance of the necropsy for the detection of possible tumors at any site is vital to carcinogenesis experiments". Qualified and well-supervised personnel are absolutely essential.

3.4 The subcontractor must have personnel available for weekend duty to necropsy any dead or moribund animals.

4. QUALITY CONTROL

4.1 The credentials of all persons to be engaged for pathology responsibilities or histologic duties in the National Cancer Institute Carcinogen Bioassay Program who are not board-certified or registered shall be submitted by the subcontractor for review and approval by program management.

Subject:	Date:	Sheet	Of
CARCINOGEN BIOASSAY PATHOLOGY PERSONNEL		2	2

5. PACKAGING

N/A

6. REFERENCE DOCUMENTS

6.1 Sontag, J.M., N.P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U.S. Department of Health, Education and Welfare, NIH 76-801.

6.2 Request for Proposal 76-S-12, Carcinogen Bioassay Program, Due Date June 15, 1976, Tracor Jitco, Inc., Rockville, Maryland.

Carcinogen Bioassay Program — Specification No. CBO-4

Type: GOOD ANIMAL CARE LABORATORY PRACTICE					**Sheet** 1	**Of** 4
Subject: SAFETY OF ANIMAL CARE PERSONNEL						
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**	

1. SCOPE

This specification covers the requirements needed to protect laboratory animal care personnel from potential hazards - the restriction of certain personnel into particular facilities, the use of protective clothing and equipment, the personal hygiene aspect and the enforcement of an occupational health program.

2. APPLICABLE DOCUMENTS

2.1 NCI Safety Standards for Research Involving Chemical Carcinogens. 1975. Department of Health, Education and Welfare, NIH 76-900.

2.2 OSHA Standard for Carcinogens, Federal Register, Vol. 39, No. 20, Jan., 29, 1974.

2.3 OSHA Standard for Carcinogens, Part II, Federal Register, Vol. 41, No. 163, p. 35184, August 20, 1976.

3. REQUIREMENTS

Personnel should receive adequate animal care and personal hygiene training and instruction as to the proper operating procedure.

3.1 Personnel restrictions.

3.1.1 Access to the animal facilities should be restricted to those individuals essential to their operation.

3.1.2 Personnel whose medical condition, e.g., depressed immune response, pregnancy, and steroid or cytotoxic drug treatment, may make them unusually susceptible to the possible harmful effects of a test agent should be excluded from any area where accidental exposure could occur.

3.1.3 Individuals who are allergic to laboratory animals should not be exposed to them unless adequately protected and approval has been given by the medical or safety officer.

3.2 Use of protective clothing and equipment.

3.2.1 A complete change of clean working clothes should be provided daily and should include a fully fastened laboratory suit or jumpsuits, gloves, boots, and head cover.

3.2.2 Clothing contaminated by chemical carcinogens shall be decontaminated before being sent out for laundering or it shall be disposed of immediately after an overt exposure.

3.2.3 An appropriate face mask or respirator should be worn as protection against dust, mists, or fumes.

3.2.4 The protective clothing should not be worn outside the work area.

3.2.5 Suitable facilities should be available for storage of street clothing during the workday.

3.3 Personal hygiene.

3.3.1 There shall be no eating, drinking, smoking, application of cosmetics, or storage of food within animal room or in areas where chemical carcinogens are used.

3.3.2 Showering or a surgical scrub to the elbows, prior to entry into the clean area is recommended.

3.3.3 Face and neck skin surfaces should be hygienically cleaned.

3.4 Occupational health program.

3.4.1 An occupational health program is mandatory for personnel working in laboratory animal facilities and for other personnel with significant animal contact.

3.4.2 It should include preplacement and periodic physical examinations.

3.4.3 The specific occupational hazards that may exist should be recognized.

3.4.4 An immunization schedule appropriate to the animal care program should be developed.

3.4.5 Zoonosis surveillance should be carried out.

3.4.5.1 Keep a permanent case record of individual work assignments.

3.4.5.2 Retain records concerning bite wounds and occurrence of any unusual illness.

3.4.5.3 Instruct personnel to notify their supervisor of suspected health hazards.

3.4.5.4 Obtain and store individual preplacement and post-employment sera for future diagnostic purposes.

4. QUALITY CONTROL

4.1 Periodic inspection to assure that safety regulations have been carried out.

5. REFERENCE DOCUMENTS

5.1 Sontag, J.M., N.P. Page, and V. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

5.2 Guide for the Care and Use of Laboratory Animals. 1974. U.S. Department of Health, Education and Welfare, NIH 74-23.

5.3 Procurement Specification IX. Defined Laboratory Rodents and Rabbits. 1973. Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D. C.

5.4 Long-Term Holding of Laboratory Rodents. 1976. ILAR News XIX (4), L20, L21.

6. NOTES

6.1 Definitions.

6.1.1 CHEMICAL CARCINOGEN is a chemical that has been demonstrated to cause tumors in mammalian species by induction of a tumor type not usually observed; or by induction of an increased incidence of tumor type normally seen, or by its appearance at a time earlier than would be otherwise expected.

Carcinogen Bioassay Program

Specification No. CBO-4

Subject:	Date:	Sheet	Of
SAFETY OF ANIMAL CARE PERSONNEL		4	4

6.1.2 DECONTAMINATION is the safe removal of a chemical carcinogen from a contaminated item.

6.1.3 DISPOSAL is the safe elimination of a chemical carcinogen from the general environment by inactivation, degradation, destruction, or other appropriate method.

6.1.4 GLOVES are covers to protect the hands of a worker against contact with or exposure to chemical carcinogen.

6.1.5 PROTECTIVE CLOTHING is clothes designed to protect a worker against contact with or exposure to a chemical carcinogen.

6.1.6 PROTECTIVE EQUIPMENT is equipment in addition to protective clothing and gloves, such as a face mask or a respirator, that is designed to protect a worker against contact with or exposure to chemical carcinogen.

Carcinogen Bioassay Program

Specification No. CBO-5

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 3

Subject:

PREPARATION FOR SHIPMENT AND TRANSPORTATION OF LABORATORY ANIMALS

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers the precautions taken during transportation of experimental animals between facilities to minimize contamination and alteration of the behavior and physiologic status of the animals.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Shipping containers

3.1.1 Shipping containers must be made of new materials. The materials should be nontoxic and impervious to moisture.

3.1.2 All inner surfaces of containers should be wire-screened when the materials call for it.

3.1.3 Twenty-five to thirty percent of the surface areas must be open and covered by filter.

3.1.4 Ventilation openings should be decreased during severe cold weather.

3.1.5 The bedding in the shipping containers must be clean and adequate to assure sanitation and comfort.

3.1.6 Shipping containers should be sterilized prior to packing.

3.2 Transportation vehicle and its environmental control

3.2.1 Vehicles used must be mechanically sound and equipped to provide fresh air without injurious draft to all animals being transported.

3.2.2 Exhaust from the vehicle's engine should not have ingress to the animal cargo space.

3.2.3 Animal cargo space must be kept clean.

Subject: PREPARATION FOR SHIPMENT AND TRANSPORTATION OF LABORATORY ANIMALS	**Date:**	**Sheet** 2	**Of** 3

3.2.4 The shipper should be conservative in determining the number of animals to be placed in a shipping container. The animals should have sufficient space to turn freely, stand erect, and lie naturally.

3.2.5 During periods of high outdoor temperature, limitations upon the number of animals per shipping container are especially important.

3.2.6 Vehicles must be sanitized before loading.

3.2.7 Vehicles should maintain a temperature suitable for the animals by air-conditioner, heater or other devices.

3.3 Feed and water for animals during shipment.

3.3.1 Each shipping container must have sufficient food and water to maintain the animals for approximately double the time period normally estimated for transit from consignor to consignee.

3.4 Qualification of the driver of the vehicle.

3.4.1 The driver should be qualified to handle and care for the laboratory animals being transported and to provide needed services in emergencies.

3.4.2 This qualification may consist of either completion of an approved animal care technician course or equivalent practical experience in animal care.

3.5 Schedule

3.5.1 Schedule of shipments must be planned to minimize the amount of time that animals are in transit. For example, shipments should be scheduled for normal working days, usually Mondays through Thursdays, since delivery and reception are often unreliable on Fridays, holidays and weekends.

3.6 Shipping labels

3.6.1 Shipping labels should contain the following information: origin of shipment; name, address and zip code and telephone number of the consignee; purchase order number, if available; date of shipment; kind and total number of animals and number of containers per shipment; instruction for special handling, feeding or watering, if required; delivery ticket for signature of consignee acknowledging receipt of shipment.

Carcinogen Bioassay Program Specification No. CBO-5

Subject: PREPARATION FOR SHIPMENT AND TRANSPORTATION OF LABORATORY ANIMALS	**Date:**	**Sheet** 3	**Of** 3

4. QUALITY CONTROL

4.1 The project management should check to assure that the transport requirements have been carried out.

4.2 Shipping containers and transportation vehicles must be inspected to see they meet the specifications set forth.

4.3 Acceptance of animals at the purchaser's facilities depends upon freedom from overt signs of disease and parasitism, scars, wounds, lesions and abnormal physical and behavioral characteristics.

4.4 Freedom from certain microbial organisms and ecto and endo parasites should be delineated by purchasers.

5. PACKAGING

N/A

6. REFERENCE DOCUMENTS

6.1 Procurement Specification IX. Defined Laboratory Rodents and Rabbits. 1973. Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D. C.

6.2 Long-Term Holding of Laboratory Rodents. 1976. ILAR News XIX (4) L20, L21.

6.3 Procurement Specification VII. Rodents. 1969. Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D. C.

Carcinogen Bioassay Program   Specification No. CBO-6

Type: GOOD ANIMAL CARE LABORATORY PRACTICE				**Sheet** 1	**Of** 3
Subject: RECEIPT AND QUARANTINE OF LABORATORY ANIMALS					
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This practice covers examination and caging of animals upon receipt from supplier through quarantine at the laboratory.

2. APPLICABLE DOCUMENTS

2.1	Specification No. CBM-6	Animal Cages and Cage Filters
2.2	Specification No. CBO-7	Weighing of Laboratory Animals
2.3	Specification No. CBO-8	Examination of Animals for General Health
2.4	Specification No. CBO-9	Examination of Animals for Parasites
2.5	Specification No. CBO-21	Sacrifice of Living Animals
2.6	Specification No. CBO-17	Maintenance and Optimal Environmental Conditions for Laboratory Animals
2.7	Specification No. CBO-19	Disinfection of Animal Laboratory Rooms

2.8 Code of Federal Regulations, Title 9, Chapter 1, Subchapter A. Animal Welfare. Parts 1, 2 and 3, May, 1972.

3. REQUIREMENTS

3.1 Examination upon receipt

3.1.1 Animals shall be received, in their unopened shipping containers, in the designated quarantine area.

3.1.2 Discard substandard animals on receipt for size, health or other reasons.

Minimum Acceptable Size:

Mice____________g

Rats____________g

Examine all animals for general health. Sacrifice a random sample of _________ animals and examine for parasites. Palpate all animals and discard any with an abnormality.

Carcinogen Bioassay Program Specification No. CBO-6

Subject: RECEIPT AND QUARANTINE OF LABORATORY ANIMALS	**Date:**	**Sheet** 2	**Of** 3

3.2 Caging before distribution for test

A shipment may be caged together during quarantine, acute toxicity test and repeated dose study according to the weight-space requirements in the following chart:

SPECIES	WEIGHT	FLOOR AREA/ANIMAL (SQUARE)	HEIGHT*
Mouse	Up to 10 g	39 cm (6 in)	12.7 cm (5 in)
	10-15 g	52 cm (8 in)	12.7 cm (5 in)
	16-25 g	77 cm (12 in)	12.7 cm (5 in)
	Over 25 g	97 cm (15 in)	12.7 cm (5 in)
Rat	Up to 100 g	110 cm (17 in)	17.8 cm (7 in)
	100-200 g	148 cm (23 in)	17.8 cm (7 in)
	201-300 g	187 cm (29 in)	17.8 cm (7 in)
	Over 300 g	258 cm (40 in)	17.8 cm (7 in)

* Height means from the resting floor to the cage top.

3.3 Physical facilities of quarantine area.

3.3.1 The quarantine area should be located in rooms physically separated from existing testing areas. Separate rooms should be provided for each species.

3.3.2 Except for relaxed caging requirements prior to distribution, physical conditions during quarantine shall be of the same quality as that provided animals under test.

3.3.3 If an epizootic disease or parasitic infection if found among the animals upon arrival, or at any time during quarantine, the entire shipment should be discarded and the room disinfected prior to the receipt of additional animals.

3.4 Quarantine period

Animals should be quarantined for a minimum of seven days.

3.5 Reexamination of animals.

3.5.1 At the end of the quarantine period, the animals should be reexamined for health (and palpated) and any additional substandard ones discarded.

3.5.2 If a sufficient number of healthy animals to satisfy test protocol requirements are on hand after reexamination, they may be distributed for testing. If the number is insufficient, a new supply of animals may need to be obtained and the quarantine and examination repeated.

Subject: RECEIPT AND QUARANTINE OF LABORATORY ANIMALS	**Date:**	**Sheet** 3	**Of** 3

3.6 Disposal of animals dead on receipt or during quarantine.

3.6.1 All procedures involved in the disposal of dead animals shall be in conformance with Federal, State, and local laws and regulations pertaining to pollution control and protection of the environment.

3.6.2 Waste cans for use in removal of dead animals should be equipped with leakproof disposable liners and tight-fitting lids.

4. QUALITY CONTROL

4.1 Shipments containing dead, moribund, or unsatisfactory animals must be reported immediately to the program management and in writing to the animal-supply house concerned, with a copy to the program management.

4.2 Results of examination for parasites of individual animals in the sample sacrificed, including all negative findings, shall be recorded in a bound laboratory notebook by the clinician performing the examination and witnessed by the laboratory supervisor. It shall be the responsibility of the laboratory supervisor to verify that a complete record has been made for each shipment within ______ days of receipt of the shipment.

4.3 The number of animals entering quarantine, length of quarantine, and the number distributed for testing, with a tabulation by cause of all discards, shall be entered in a bound laboratory notebook by the responsible technician and witnessed by the laboratory supervisor. This record shall be available for audit and analysis.

4.4 If occurrence of an epizootic disease has been reported, it shall be the responsibility of the laboratory supervisor to verify in the quarantine laboratory notebook that the quarantine area has been disinfected within _____ hours of the detection of the disease and removal of the affected shipment.

5. N/A

6. REFERENCE DOCUMENTS

6.1 Sontag, J.M., N.P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U.S. Department of Health, Education and Welfare, NIH 76-801.

6.2 Guide for the Care and Use of Laboratory Animals. 1974. U.S. Department of Health, Education and Welfare, NIH 74-23.

6.3 Guide to Infectious Diseases of Mice and Rats, Institute of Laboratory Animal Resources, ISBN 0-309-01914-1.

Carcinogen Bioassay Program Specification No. CBO-7

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 1
Subject: WEIGHING OF LABORATORY ANIMALS		

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers weighing of laboratory animals for caging, randomization, recording weight change during bioassay, and other operations in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

2.1	Specification No. CBO-6	Receipt and Quarantine of Laboratory Animals
2.2	Specification No. CBO-10	Randomization, Assignment, and Identification of Animals
2.3	Specification No. CBP-2	Repeated-Dose Test, Carcinogen Bioassay Program
2.4	Specification No. CBP-3	Sub-chronic Test, Carcinogen Bioassay Program
2.5	Specification No. CBP-4	Chronic Carcinogenicity, Carcinogen Bioassay Program

3. REQUIREMENTS

3.1 All animals used in the Carcinogen Bioassay Program shall be weighed individually at times indicated: at time of receipt, at time of assignment to treatment groups, and periodically during chronic studies.

3.2 Weight shall be determined to the nearest gram using an appropriate animal weighing scale.

4. QUALITY CONTROL

4.1 Balances employed for determining animal weight shall be recalibrated at least monthly and calibration data recorded in a bound notebook and signed by responsible personnel.

4.2 Supervisors shall be responsible for making certain that all animal weights are accurately determined and recorded to insure validity of the bioassay test results.

5. PACKAGING

Not applicable.

Carcinogen Bioassay Program

Specification No. CBO-8

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 2

Subject:

EXAMINATION OF LABORATORY ANIMALS FOR GENERAL HEALTH

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers the observation of laboratory animals for signs of diseases and ways of monitoring the general health of the animals.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Observation. All animals should be observed regularly by properly qualified personnel for signs of diseases. Animal care should be under direction of veterinarians with specialized training and experience in laboratory animal medicine.

3.1.1 Sick or moribund animals or animals found dead should be removed from the colony, and an adequate number should be examined by laboratory procedures (including pathology) to determine the cause of the observed signs or death.

4. QUALITY CONTROL

4.1 Monitoring

4.1.1 Routine methods. At regularly scheduled intervals, water bottles and feces should be cultured in order to determine whether the predominant organisms present are similar or identical to those previously established and that pathogens are not present.

4.1.2 Detailed methods. At regularly scheduled intervals, normal-appearing animals should be removed from the colony for laboratory tests.

4.1.2.1 Serum samples should be obtained and tested for antibodies to murine viruses.

4.1.2.2 Bacteria, mycoplasma, protozoa, and metazoa should be identified, if present.

4.1.2.3 Tissues or organs should be examined histologically to determine the presence or absence of lesions.

Subject:	**Date:**	**Sheet**	**Of**
EXAMINATION OF LABORATORY ANIMALS FOR GENERAL HEALTH		2	2

4.1.3 Record keeping. Daily records shall be maintained on morbidity, mortality, and laboratory findings by room, species, and strain. This information should be reviewed weekly.

5. PACKAGING

Not applicable here.

6. REFERENCE DOCUMENTS

6.1 Procurement Specification IX. Defined Laboratory Rodents and Rabbits. 1973. Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D. C.

Carcinogen Bioassay Program

Specification No. CBO-9

Type:	**Sheet**	**Of**
GOOD ANIMAL CARE LABORATORY PRACTICE	1	1

Subject:

EXAMINATION OF RODENTS FOR PARASITES

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers examination of rodents for parasites.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Parasitology.

3.1.1 Routine methods. Microscopic examination of specimens obtained from fresh feces by concentration procedures and scotch tape impressions of the perianal region from representative animals should be examined for the presence of parasitic ova.

3.1.2 Detailed methods.

3.1.2.1 At the time of sacrifice, in addition to routine methods described above, urine should be examined microscopically for nematode eggs, and the intestinal tract, cecum, and bladder opened and examined with appropriate magnification for internal parasites.

3.1.2.2 In addition, histologic examination of the tissue and organs will assist in determining whether selected protozoan or metazoan parasites are present. Special attention and selective strains are recommended for the lower respiratory tract and brain for Pneumocystis and Nosema, respectively.

4. QUALITY CONTROL

4.1 Refer to the Diagnostic Guide (Section I) and Disease Outlines (Sect 11) of "A Guide to Infectious Diseases of Mice and Rats", National Academy of Sciences, for descriptions of clinical and pathologic features of diseases plus appropriate diagnostic procedures.

4.2 Positive and negative findings shall be reported for each animal examined. It shall be the responsibility of laboratory supervision to monitor the examination to assure its completeness and correctness.

Carcinogen Bioassay Programs Specification No. CBO-10

Type: GOOD ANIMAL CARE LABORATORY PRACTICE				**Sheet** 1	**Of** 4
Subject: RANDOMIZATION, ASSIGNMENT, AND IDENTIFICATION OF ANIMALS					
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This practice covers the age of animals assigned to an experiment, the use of random numbers to select animals by weight distribution for assignment to experimental groups, and the unique identification of individual animals.

2. APPLICABLE DOCUMENTS

2.1 Specification No. CBO-7 Weighing of Animals

3. REQUIREMENTS

3.1 Animal species, strain and sex

Experimental groups are composed of animals of the same species, strain and sex, each group being dealt with identically.

3.2 Age of animals

3.2.1 At the start of the chronic study, animals should be no older than six weeks and, if possible, weanlings.

3.2.2 All animals assigned to a study should be within two to three days of the same age. This is assured by specifying age limits at the time of animal procurement and using only animals from the same shipment in an experiment.

3.2.3 If it has been necessary to replace animals lost from a shipment upon receipt or during quarantine, the animals should be segregated initially by shipment. A randomization procedure is used to ensure that there will be a proportionate number of animals from each shipment and from each weight distribution group (see next section) in each of the experimental groups.

3.3 Weight of animals

3.3.1 The animals should be initially segregated into equal weight distribution groups according to the following table.

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RANDOMIZATION, ASSIGNMENT, AND IDENTIFICATION OF ANIMALS		2	4

Weight Distribution Groups

Species	Weight
Mouse	Up to 10 g
	11-15 g
	16-25 g
	Over 25 g
Rat	Up to 100 g
	101-200 g
	201-300 g
	Over 300 g

3.3.2 After segregation by weight, animals are to be divided into experimental groups using a randomization procedure to assure that a proportionate number of animals from each weight distribution group are included in each experimental group.

3.4 Distribution in sub-chronic and chronic studies

3.4.1 Distribute animals from the outset of the studies as if they were in the upper weight range in above table.

3.4.2 No cage should contain more than five animals.

3.4.3 As animals die or are sacrificed, surviving animals should not be combined or redistributed among the cages.

3.5 Randomization procedure

3.5.1 Experimental groups must be balanced, that is, each group must contain an equal number of animals and representation of initially segregated weight groups (or age groups, if necessary) in each experimental group must be proportional to the size of the initially segregated groups.

3.5.2 If segregation by age was required, first make the separation by age and then by weight within each age group. If each experimental group is to contain 50 animals, the total number of animals in all the initial age/weight groups together must be at least 50 times the number of experimental groups required by the experiment design.

3.5.3 The randomization procedure is followed separately for each age/weight group.

3.6 Identification of animals

Each animal should be uniquely identified at the time it is assigned to a sub-chronic or chronic experimental group by toe clipping, ear notching or other appropriate method.

4. QUALITY CONTROL

A record of all shipments included in a study, weight groupings, randomization and assignment to experimental groups and animal identifications should be made in a bound laboratory notebook by the technician and witnessed by the laboratory supervisor.

4.1 Determine the number of animals available in each age/weight group.

4.2 Divide the number in each of these groups by the number of experimental groups required. This gives the proportional number of animals for each initial group to be included in each experimental group. Because total available animals may be more than exactly 50 times the number of experimental groups, or because the numbers in the initial groups may not be exactly divisible by the number of experimental groups, it may be necessary to adjust the dividend slightly to add up to a total of exactly 50.

4.3 Temporarily number the animals in the first age/weight group consecutively. Select a random starting place in a table of random numbers. Read from the table, omitting 000, numbers larger than the total number in the group, and repeats. Arrange these numbers in successive sets the size of the proportional number determined in the preceding section. These sets are assigned to the respective experimental groups.

4.4 Repeat the procedure of the preceding section for each size/weight subgroup.

4.5 Example of Randomization Procedure. Suppose that the experiment design calls for five experimental groups of 50 animals each, that 200 satisfactory animals are available from a replacement shipment. Assume the following weight distribution and calculate the proportional number as shown.

Original Shipment	No. of Animals	Proportional No.
Weight Group 1	30	6
Weight Group 2	80	16
Weight Group 3	70	14
Weight Group 4	20	4

Replacement Shipment	No. of Animals	Proportional No.
Weight Group 1	10	2
Weight Group 2	20	4
Weight Group 3	15	3
Weight Group 4	5	1
Grand Totals	250	50

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RANDOMIZATION, ASSIGNMENT AND IDENTIFICATION OF ANIMALS		4	4

Number the animals in the first group from 1 to 30. Enter a random table and list numbers from 001 to 030 as they appear, omitting repeats, until five sets of six numbers each have been obtained. These sets are assigned to the five experimental groups. Repeat this procedure for each group in the above table.

Carcinogen Bioassay Program

Specification No. CBO-11

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 3

Subject:

STORAGE OF FEED, BEDDING, AND EQUIPMENT FOR LABORATORY ANIMALS

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers storage of feed, bedding, and equipment used in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Date of manufacture of all feed supplies shall be checked upon receipt. Products delivered 90 days or more after manufacture shall not be accepted.

3.2 Feed and bedding shall be stored in a clean area and protected from spoilage or deterioration and infestation or contamination by vermin. A continuous pest control program is essential. Containers shall be stored off the floor on pallets, racks, or carts. The area shall be physically separated from refuse areas.

3.3 Feed shall be stored in receptacles with tightly fitting lids or covers which can be sanitized before reuse, or in original containers as received from the supplier. The storage area shall be cool (10° C or less), dry, and airy.

3.4 Washed/sanitized equipment shall be stored in a clean area free of vermin.

3.5 All supplies of feed and bedding as well as equipment in storage shall be carefully protected against contamination by pesticides. Pesticides shall not be used inside buildings unless specifically agreed to by program management.

4. QUALITY CONTROL

4.1 Date of manufacture and delivery date of all feed shipments shall be recorded in a bound notebook maintained for later consideration and signed by personnel receiving same.

Carcinogen Bioassay Program Specification No. CBO-11

Subject: STORAGE OF FEED, BEDDING, AND EQUIPMENT FOR LABORATORY ANIMALS	**Date:**	**Sheet** 2	**Of** 3

4.2 The shelf life of all feed lots shall be checked as used to avoid feeding outdated rations to test animals.

4.3 Temperature in the feed storage area shall be recorded continuously by an automatic recording thermometer. Temperature recordings shall be inspected daily and adjustments made when necessary to maintain a temperature of 10° C or less. All charts shall be dated, signed, and filed for audit by program management.

4.4 The automatic temperature recorder shall be recalibrated at least monthly and data recorded and signed by technical personnel performing the work.

4.5 All storage areas shall be inspected weekly for the presence or evidence of vermin and appropriate action taken when necessary.

4.6 Feed in containers found open during inspections shall not be used.

4.7 If pesticides are used in the animal facility, supplies of feed and bedding shall be analyzed at monthly intervals. Results of all analyses shall be reported immediately to program management who will notify the bioassay laboratory of any lots unsuitable for use.

5. PACKAGING

5.1 Feed shall not be shipped or stored in plastic containers.

5.2 Feed containers must be sealed to prevent contamination during transit. Broken or repaired containers of feed shall be rejected.

6. NOTES

6.1 Feed that is older than 90 days may be unsatisfactory due to loss of essential nutrients.

6.2 Plastic materials are unsatisfactory for feed containers since they melt during autoclaving and may, under certain environmental conditions, provide conditions favorable for the growth of molds.

7. REFERENCE DOCUMENTS

7.1 Long-Term Holding of Laboratory Rodents. 1976. ILAR News XIX (4), L20, L21.

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7.2 Code of Federal Regulations, Title 9, Chapter 1, Subchapter A. Animal Welfare. Parts 1, 2 and 3, May, 1972.

7.3 Guide for the Care and Use of Laboratory Animals. 1974. U.S. Department of Health, Education and Welfare, NIH 74-23.

7.4 The UFAW Handbook on the Care and Management of Laboratory Animals. 1972. 4th edition. UFAW Staff (eds.). Churchill Livingstone, Edinburgh and London.

7.5 Tracor Jitco Subcontract for Carcinogen Bioassay with Industrial Bio-Test Laboratories, Inc., Subcontract No. 76-33-106002, April 19, 1976.

7.6 Tracor Jitco Subcontract for Carcinogen Bioassay with Battelle-Columbus Laboratories, Subcontract No. 76-34-106002, April 8, 1976.

Carcinogen Bioassay Program Specification No. CBO-12

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 3

Subject: DOSE PREPARATION AND ANALYSIS FOR CHEMICALS TO BE ADMINISTERED BY PROCEDURES OTHER THAN INHALATION

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers mixing of the test chemical with feed or other carrier, storage, and analysis of the mixture for concentration, homogeneity, and stability.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 A procedure for mixing test chemical with feed or vehicle which will insure homogeneity of dose preparations shall be developed by the analytical subcontractor prior to bioassay.

3.2 The stability and storage parameters of each test chemical also shall be determined by the analytical subcontractor prior to its bioassay.

3.3 The bioassay laboratory shall follow the mixing procedure, storage conditions, and frequency of dose preparation recommended by the analytical subcontractor. Any difficulties with or deviations from these procedures shall be reported promptly to program management.

3.4 The bioassay laboratory shall analyze all dosage mixtures by procedures developed by the analytical subcontractor.

3.4.1 A sample of each dose-feed mixture and stock liquid mixture (highest level only for the latter) during the chronic study at time of mixing shall be stored in individual labelled and sealed containers at 5° C or lower.

3.4.2 One-eighth of the chronic test samples, selected randomly and blind to dosage preparation personnel, shall be analyzed by the bioassay laboratory immediately after mixing or no more than one week later.

3.4.3 During the sub-chronic study, a single sample at each level will be analyzed to demonstrate efficiency of the mixing procedure and of the analytical method.

Subject: DOSE PREPARATION AND ANALYSIS FOR CHEMICALS TO BE ADMINISTERED BY PROCEDURES OTHER THAN INHALATION	**Date:**	**Sheet** 2	**Of** 3

3.4.4 Analysis shall consist of determination of the concentration of test chemical in dose mixture to insure accuracy of weighing and mixing processes as well as stability of the chemical.

3.4.5 Analytical methodology normally will consist of re-isolation of test chemical from the dose mixture and spectroscopic or chromatographic analysis by a procedure developed for each test chemical individually.

3.4.6 Analytical values which differ from that of the expected concentration by more than 10% shall be considered out of tolerance and shall not be given to test animals (cause of deviation will be discussed in the analytical report). Replacement preparations shall be analyzed immediately. Unanalyzed samples shall be discarded 90 days after mixing.

3.4.7 Analytical results will be reported to the Principal Investigator immediately. Copies of results will be submitted to program management as indicated on section Reports.

3.5 An inventory of each dosage mixture shall be maintained on a current basis. Preparation date, amount prepared, usage dates, amounts used, and names of responsible personnel shall be included.

3.6 Any instability of chemical in dose mixture shall be reported immediately to program management.

4. QUALITY CONTROL

4.1 Prior to the bioassay, the analytical subcontractor shall document:

4.1.1 Homogeneity of dose preparation of test chemical according to the mixing procedure developed.

4.1.2 Stability of the test chemical under conditions of mixture with feed or vehicle and storage.

4.2 All analytical instruments used by the analytical subcontractor and bioassay laboratory shall be re-calibrated monthly. All recalibration data shall be recorded in a bound notebook maintained for the purpose and signed by personnel and supervisor involved.

4.3 All temperature charts of refrigerated storage for dose preparations throughout the course of study shall be dated, signed, and filed for audit. Thermometers shall be re-calibrated monthly and data filed as in 4.2 above.

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5. PACKAGING

Not Applicable

6. REFERENCE DOCUMENTS

6.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

Carcinogen Bioassay Program Specification No. CBO-13

Type: GOOD ANIMAL CARE LABORATORY PRACTICE				**Sheet** 1	**Of** 2
Subject: FEEDING OF LABORATORY ANIMALS					
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers the feeding procedures and requirements for feeders.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 FEEDING PROCEDURES

3.1.1 Provide feed as often as necessary, but not less than once weekly, to assure an adequate supply of fresh rations.

3.1.2 Supply sanitized feeder at least once weekly.

3.1.3 Analyze the feed for pesticide, mycotoxin, and industrial contaminants periodically.

3.1.4 Sterilize feed whenever practical and consistent with the disease control program.

3.1.5 Care should be taken that nutrients are not degraded or the palatability of the feed altered.

3.2 REQUIREMENTS FOR FEEDER

3.2.1 Feeder shall be accessible to all animals.

3.2.2 Feeder shall be located so as to minimize contamination by excreta.

3.2.3 Feeder shall be durable and kept clean.

3.2.4 Sanitize feeder at least once every two weeks.

3.2.5 Discard disposable feeder after each feeding.

4. QUALITY CONTROL

4.1 Nutrient Analysis. Collect random feed samples quarterly and analyze in accordance with the AOAC methods of analysis (Association of Official Analytical Chemists, 1975).

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4.2 Retention of Feed Samples.

4.2.1 Retain a 500 to 800 g sample from each production batch of feed used.

4.2.2 Store in freezer or in sealed containers placed in a cool, dry, area, for the duration of the experiment invovled.

4.3 Microbiologic Monitoring

It is recommended that periodic sterilizer runs be monitored to assure that vegetative forms of microorganisms have been killed. This may be most easily accomplished by placing a filter paper strip impregnated with Escherichia coli in the center of load. The strip is then incubated in a suitable medium and examined for growth. Food may be held in a clean storage area until culture results are available.

4.4 A program of periodic assay for the chemical contaminants that may interfere with results of a particular study is recommended.

4.4.1 If unacceptable concentrations are detected, a change in ration or source may be in order.

4.5 A continuing pest control program is essential in the food storage area.

5. PACKAGING

N/A

6. REFERENCE DOCUMENTS

6.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

6.2 Long-Term Holding of Laboratory Rodents. 1976. ILAR News XIX (4), L20, L21.

6.3 Code of Federal Regulations, Title 9, Chapter 1, Subchapter A. Animal Welfare. Parts 1, 2 and 3, May, 1972.

Carcinogen Bioassay Program Specification No. CBO-14

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	Sheet 1	Of 3

Subject: GENERATION AND ANALYSIS OF TEST ATMOSPHERES OF CHEMICALS EVALUATED BY THE INHALATION METHOD PROGRAM

Approved:	Proj.	Q.C.	Lab	Other	Date

1. SCOPE

This specification covers generation of test atmosphere of chemicals studied by the inhalation method in the Carcinogen Bioassay Program together with analytical and control procedures employed.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Gases shall be introduced into the main chamber air supply by means of a pressure regulator in combination with a flowmeter and mixed with the air supply by turbulence in the mixing chamber prior to actual introduction into the exposure chamber.

3.2 Inhalation test preparations of liquids shall be generated by bubbling clean (charcoal and HEPA-filtered), dry air (-40° C dewpoint) through all-glass impingers containing the test chemical.

3.3 The concentration of test chemical in exposure chamber shall be monitored continuously by means of an automated sampling system. Sampling from a single port will suffice if preliminary data demonstrated uniform concentration of test chemical throughout the chamber.

Chamber concentration of the agent shall be calculated also from data on mass transfer from generator and flow rate through chamber as a backup method.

3.4 The exposure chamber atmosphere shall be maintained at a temperature of $23.3^\circ \pm 1.1^\circ$ C ($74^\circ \pm 2^\circ$ F) and $50 \pm 5\%$ relative humidity. Chamber pressure shall be negative (approximately 0.5-1.0 cm H_2O) in relation to the room pressure. Air flow rate, temperature, and humidity shall be monitored continuously and recorded. Air pressure shall be recorded at least daily.

3.5 Chamber temperature shall be determined at two locations at least by remote sensors. Air flow rates shall be controlled by

Subject: GENERATION AND ANALYSIS OF TEST ATMOSPHERES OF CHEMICALS EVALUATED BY THE INHALATION METHOD PROGRAM	**Date:**	**Sheet** 2	**Of** 3

precision rotameters, calibrated pressure-drop orifices, and mass flowmeters.

3.6 The exposure chamber shall be equipped with an emergency alarm system for detection of all significant deviations from test limits for air flow, chamber pressure, temperature, and test chemical concentration. Laboratory personnel also shall carefully observe all chamber instruments throughout the study.

3.7 Uniformity of test chemical throughout the exposure chamber shall be documented during development of the exposure technique and again at beginning of the bioassay with animals in the chamber.

3.8 The exposure chamber atmosphere shall be checked for the absence of test chemical during non-exposure periods prior to the bioassay and at intervals during the study. Appropriate action shall be taken to insure the absence of the agent during non-exposure periods if necessary.

3.9 If stability of test chemical is questionable, the chamber atmosphere shall be tested for known or suspected degradation products at intervals during the study.

3.10 When liquid chemicals are tested in the form of a molecular vapor rather than as an aerosol, the test atmosphere shall be tested by photometric or other appropriate means to insure the absence of significant concentration of particulates of the agent.

3.11 Exhaust test atmospheres from the chamber shall first be passed through an appropriate scrubber for the test chemical and then through filters in the common exhaust vent to an outside stack, and finally through a second air scrubber.

Effluent air stacks shall be sampled daily for air concentration of the test chemical.

4. QUALITY CONTROL

4.1 The following equipment shall be calibrated at least monthly by qualified technicians: pressure regulators, flowmeters, temperature sensors, rotameters, photometers, pressure-drop orifices, and automated sampling system for determination of test chemical concentration. All calibration data shall be dated and recorded in a bound notebook maintained for the purpose and signed by personnel and supervisors involved.

Carcinogen Bioassay Program Specification No. CBO-14

Subject: GENERATION AND ANALYSIS OF TEST ATMOSPHERES OF CHEMICALS EVALUATED BY THE INHALATION METHOD PROGRAM	**Date:**	**Sheet**	**Of**
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4.2 The emergency alarm system for indicating significant deviation from test parameters shall be tested weekly and results recorded and signed as in 4.1 above.

4.3 Bioassay supervisors shall carefully supervise all steps in the bioassay study to make certain that all procedures are in compliance with Subcontract regulations and "NCI Guidelines for Carcinogen Bioassay in Small Rodents" including Appendix C - Safety Standards for Research Involving Chemical Carcinogens.

4.4 All pertinent data in "NCI Guidelines" - Appendix F - Carcinogen Bioassay Information - shall be collected and reported in accordance with the Carcinogenesis Bioassay Data System (CBDS) procedures.

5. PACKAGING

Not Applicable

6. REFERENCE DOCUMENTS

6.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

Carcinogen Bioassay Program

Specification No. CBO-15

Type:

GOOD ANIMAL CARE LABORATORY PRACTICE

Sheet 1 **Of** 3

Subject:

WATERING OF LABORATORY ANIMALS

Approved:	Proj.	Q.C.	Lab	Other	Date

1. SCOPE

This specification covers the operations in the watering of laboratory animals and the product requirements for water bottles, bottle stoppers, and sipper-tubes.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Procedure requirements for watering of laboratory animals. Watering bottles may be used although an automatic watering system is preferred.

3.1.1 Provide the animals with an adequate supply of fresh and treated water ad libitum.

3.1.2 Check to ensure that the water bottles are accessible to all animals.

3.1.3 Supply sanitized water bottles, stoppers, and sipper-tubes at least twice weekly.

3.1.4 Wash dirty water bottles promptly in a washer containing at least one cycle of water at 180°F or higher.

3.1.5 Sanitize bottle stoppers by a germicide treatment prior to washing, by boiling after washing, or by autoclaving.

3.1.6 Sterilize sipper tubes by a germicide treatment prior to washing, or by boiling after washing.

3.1.7 Fill the bottles and insert the stoppers and sipper tubes into the bottles only outside of the animal rooms.

3.1.8 Replace empty or partially full water bottles instead of refilling them.

3.1.9 Locate water bottles in a position to prevent the stoppers from being chewed by the animals.

3.1.10 Routinely examine watering device to assure their patency and use by the animals.

Subject:	Date:	Sheet	Of
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3.1.11 Kill potential pathogens carried in the water or remove them through appropriate treatment, such as sterilization, pasteurization and filtration.

3.1.12 Periodically assay drinking water for compounds that may influence experimental data (see American Public Health Association, Inc. 1971).

3.1.13 If automatic watering system is used, overall labor involved with changing, washing and filling water will be reduced. However, nozzles shall be inspected daily in order to ensure that they are functioning properly.

3.1.14 Water supply for the automatic watering device shall be treated.

3.2 Product requirements for watering equipment.

3.2.1 Water bottles. It should be made of glass or plastic with large openings and smooth surface. 500-ml capacity size is practical.

3.2.2 Sipper tubes should be made of stainless steel. The internal diameter of the sipper-tube should be 6 to 9mm and that of the terminal aperture about 3mm.

3.2.3 Stopper. One-hole-rubber-stopper is practical. It shall be protected by a suitable device to prevent gnawing by the animals.

3.2.4 Nozzles of the automatic watering system shall be capable of being rapidly disassembled for cleaning.

3.2.5 Valves of the automatic watering system shall be capable of 100 percent operative efficiency at all times.

4. QUALITY CONTROL

4.1 Microbiologic monitoring of water.

5. REFERENCE DOCUMENTS

5.1 Guide for the Care and Use of Laboratory Animals. 1974. U.S. Department of Health, Education and Welfare, NIH 74-23.

5.2 Sontag, J.M., N.P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U.S. Department of Health, Education and Welfare, NIH 76-801.

5.3 Long-Term Holding of Laboratory Rodents. 1976. ILAR News XIX (4), L20, L21.

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5.4 The UFAW Handbook on the Care and Management of Laboratory Animals. 1972. 4th edition. UFAW Staff (eds.). Churchill Livingstone, Edinburgh and London.

5.5 Workshop on Criteria for Successful Rodent Chronic Studies. National Cancer Institute, National Institutes of Health, Bethesda, Maryland, April 4-5, 1973.

Carcinogen Bioassay Program Specification No. CBO-16

Type: GOOD ANIMAL CARE LABORATORY PRACTICE

Sheet 1 **Of** 4

Subject: CHANGING OF LITTER OR BEDDING, CHANGING OF LABORATORY ANIMAL CAGES AND DISPOSAL OF WASTE

Approved:	Proj.	Q.C.	Lab	Other	Date

1. SCOPE

This specification covers changing of litter or bedding and laboratory animal cages and disposal of wastes from small rodents used in the Carcinogen Bioassay Program

2. APPLICABLE DOCUMENTS

2.1 Code of Federal Regulations, Title 9, Chapter 1, Subchapter A. Animal Welfare. Parts 1, 2, and 3, May, 1972.

2.2	Specification No. CBM-6	Laboratory Animal Cages and Cage Filters
2.3	Specification No. CBM-7	Feeders for Laboratory Animals
2.4	Specification No. CBO-4	Safety Standards for Research Involving Chemical Carcinogens

3. REQUIREMENTS

3.1 Provision shall be made for prompt removal and disposal of all food wastes from laboratory animal cages so as to minimize vermin infestation, odors, and disease hazards.

3.2 Measures must be taken to prevent molding, contamination, deterioration, or caking of feed. Uneaten fruit or vegetable supplements must not be allowed to accumulate in animal cages.

3.3 Litter or bedding shall be removed from cages as necessary to keep the animals clean and dry, and to minimize offensive odors. One to three changes per week will suffice for small rodents. Cages shall be emptied in an area set aside for the purpose away from the animal rooms.

3.4 Catch-pans for animals caged in exposure chambers shall be cleaned and relined with new absorbent paper daily.

3.5 Individually caged animals in exposure chambers shall be changed to a sanitized stainless steel wire mesh cage weekly.

3.6 Animals housed in polycarbonate cages shall be changed to a sanitized cage with fresh bedding at least twice weekly excepting when the cage population falls to one or two animals when one weekly cage change is permissible.

Subject: CHANGING OF LITTER OR BEDDING, CHANGING OF LABORATORY CAGES AND DISPOSAL OF WASTE	**Date:**	**Sheet**	**Of**
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3.7 Cages shall be chemical specific. They shall be returned to the same chemical group and dose level to prevent test chemical contamination.

3.8 All waste shall be collected and removed in a safe, sanitary manner. Cage waste may be removed to storage by vacuum. If waste cans are used, they should be made of metal or plastic and shall be leakproof and equipped with tight-fitting lids.

3.9 Waste material should be removed regularly and frequently. Waste which must be stored before final disposal shall be kept in an area maintained at a temperature of 7° C (45° F) or less. The storage area shall be separated from any other cold storage and shall be used exclusively for refuse storage. The area must be kept clean and free of vermin.

3.10 Wastes which are contaminated with chemical carcinogens shall be disposed of in accordance with applicable NCI and other Federal safety regulations (see 5.1).

3.11 Infectious wastes should be autoclaved or rendered noninfectious by other effective measures before removal from the animal facility.

3.12 Wastes which are not contaminated with carcinogens or infectious agents may be disposed of at a public incinerator or burned at the facility. Incineration shall comply with Environmental Protection Agency regulations.

3.12.1 The incinerator shall be located in such a position that stack vapors, fumes, and particulate matter will not be drawn into air-handling intake vents.

3.12.2 Stacks shall be of design which prevents emission of fly ash.

3.13 Waste disposal shall comply with all Federal, state, and municipal laws, statutes, or ordinances.

4. QUALITY CONTROL

4.1 Animal cages shall be inspected daily and litter or bedding changed as frequently as necessary, but not less than once per week, to comply with requirements set forth in Section 3 above.

4.2 Animal care personnel shall be responsible for changing animals to sanitized cages with fresh bedding as indicated in 3.5 and 3.6.

Carcinogen Bioassay Program

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Subject: CHANGING OF LITTER OR BEDDING, CHANGING OF LABORATORY ANIMAL CAGES AND DISPOSAL OF WASTE	**Date**:	**Sheet** 3	**Of** 4

4.3 Supervisors shall monitor removal and disposal of all wastes containing chemical carcinogens or infectious agents to make certain that all procedures are in compliance with applicable Federal, state, and local laws and regulations of the NCI Office of Research Safety.

4.4 Commercially available spore strips shall be included in all autoclave loads of infectious waste and subsequently cultured to monitor the efficacy of the sterilization procedure.

4.5 Data pertaining to the disposal of infectious wastes or wastes containing chemical carcinogens shall be entered in a bound notebook, dated, and signed by personnel involved and the supervisor.

5. PACKAGING

5.1 Food and other wastes contaminated with chemical carcinogens shall be placed into separate plastic bags or other suitable impermeable containers for each carcinogen, closed, sealed, and labelled with both name of carcinogen and "DANGER - - CHEMICAL CARCINOGEN", before being transported to storage or disposal area. Final disposal shall be in conformance with Federal, state, and local laws and with the Office of Research Safety Regulations.

6. REFERENCE DOCUMENTS

6.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

6.2 Request for Proposal 76-S-12, Carcinogen Bioassay, NCI Program, Due date June 15, 1976, Tracor Jitco, Inc., Rockville, Maryland.

6.3 Carcinogen Bioassay Subcontract with Industrial Bio-Test Laboratories, Inc., Subcontract No. 76-33-106002, Apr. 19, 1976.

6.4 Guide for the Care and Use of Laboratory Animals. 1974. U. S. Department of Health, Education and Welfare, NIH 74-23.

6.5 The UFAW Handbook on the Care and Management of Laboratory Animals. 1972. 4th edition. UFAW Staff (eds.). Churchill Livingstone, Edinburgh and London.

6.6 Procurement Specification IX. Defined Laboratory Rodents and Rabbits. 1973. Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D. C.

Carcinogen Bioassay Program

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Subject: CHANGING OF LITTER OR BEDDING, CHANGING OF LABORATORY ANIMAL CAGES AND DISPOSAL OF WASTE	**Date:**	**Sheet** 4	**Of** 4

6.7 Long-Term Holding of Laboratory Rodents. 1976. ILAR News XIX (4), L20, L21.

Carcinogen Bioassay Program Specification No. CBO-17

Type: GOOD ANIMAL CARE LABORATORY PRACTICE				**Sheet** 1	**Of** 7
Subject: MAINTENANCE OF OPTIMAL ENVIRONMENTAL CONDITIONS FOR LABORATORY ANIMALS					
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers temperature, ventilation, lighting, noise control, and maintenance of animal facilities used in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

2.1 Code of Federal Regulations, Title 9, Chapter 1, Subchapter A. Animal Welfare. Parts 1, 2, and 3, May, 1972.

2.2	Specification No. CBM-4	Air Filters for Carcinogen Bioassay Facilities
2.3	Specification No. CBO-4	Safety Standards for Work Involving Chemical Carcinogens
2.4	Specification No. CBM-1	Construction of the Physical Plant

3. REQUIREMENTS

3.1 Temperature and humidity

3.1.1 Each animal room or group of rooms with a common purpose shall have individual temperature and humidity controls.

3.1.2 The heating-cooling-ventilation system of the animal facility shall be sensitive to permit adjustments within ± 1°C for any temperature within the range of 18° to 29° C (65° -85°F).

3.1.3 A temperature of 23.3° C ± 1.1°C (74°F ± 2°F) shall be maintained in all mouse and rat rooms.

3.1.4 The optimum temperature for hamsters is 20-24°C. According to Federal regulations, the ambient air temperature in rooms where these rodents are quartered shall not be less than 15.6°C (60°F) or greater than 29.4°C (85°F).

3.1.5 A relative humidity of 40% ± 5% shall be maintained in all mouse and rat rooms.

3.1.6 The relative humidity for hamsters shall be 40-45%.

3.1.7 An automatic recording and alert system shall be used to monitor the ambient temperature and relative humidity in each animal room.

Subject: MAINTENANCE OF OPTIMAL ENVIRONMENTAL CONDITIONS FOR LABORATORY ANIMALS	**Date:**	**Sheet** 2	**Of** 7

3.1.8 An emergency power source shall be available with a capacity sufficient for the air conditioning and light systems of the animal facility.

3.2 Ventilation

3.2.1 Each animal room shall have 10-15 fresh-air changes per hour without drafts.

3.2.2 All air shall be adequately filtered (Specification No. CBM-4) before entering and before discharge from the animal facility.

3.2.3 The general exhaust air from areas where chemical carcinogens are used is subject to Federal regulations (Specification No. CBO-4).

3.2.4 Recirculation of exhaust air from rooms where chemical carcinogens are used is not permitted (Specification No. CBO-4).

3.2.5 Air pressure shall be adjusted so that all animal rooms are slightly positive to the "dirty" corridor and "negative" to the "clean" corridor. Rooms bordering a single access corridor shall be kept under negative pressure with respect to the corridor.

3.2.6 The animal facility and human occupancy areas shall have separate ventilation systems.

3.3 Lighting

3.3.1 Housing quarters for laboratory animals shall have ample light of good quality which is uniformly diffused throughout the area.

3.3.2 Light intensity at the cage level shall be a minimum of 100 foot-candles.

3.3.3 Examination and animal treatment areas shall have a minimum light intensity of 125 foot-candles at the work surface.

3.3.4 Continuous strip fluorescent lighting mounted flush in the ceiling is recommended. Fixtures shall be properly sealed to prevent the harboring of vermin.

3.3.5 Convenience outlets should be waterproof, recessed in walls and partitions, and located a minimum of 0.6m (2 ft) above the floor.

Subject: MAINTENANCE OF OPTIMAL ENVIRONMENTAL CONDITIONS FOR LABORATORY ANIMALS	**Date:**	**Sheet** 3	**Of** 7

3.3.6 Animal cages and other primary enclosures shall be positioned so as to protect the animals from excessive illumination.

3.3.7 A time-controlled system to provide regular diurnal lighting cycle shall be provided. Controls shall be located in the main control room.

3.3.8 Provisions must be made to provide hamsters with a lighting period of approximately 12 hours which is somewhat less than the optimum for other small rodents.

3.3.9 Light switches should be located outside each room in both clean service and evacuation corridors.

3.3.10 Lights should be serviced via a crawl space or other method which does not necessitate entering the room.

3.4 Noise control

3.4.1 Laboratory rodents, particularly mice, shall be protected from noise, especially high pitch noise (upper limits of human auditory range and beyond). Audiogenic strains must be maintained at very low noise levels.

3.4.2 All noisy operations in the animal facility, such as cage and rack cleaning and washing, etc., shall be carried out in an area separate from rooms where laboratory animals are housed.

3.4.3 Animals shall not be caged near incompatible species which disturb or distress them.

3.4.4 Carts, trucks, racks, and other moveable equipment used in animal quarters should have rubber-tired casters and rubber bumpers.

3.4.5 Concrete walls are preferred over metal or plaster construction to contain noise in animal quarters. Acoustical tile and similar materials should be used wherever possible to reduce the effect of "noise pollution" in animal rooms.

3.5 Facility maintenance

3.5.1 The operation of all animal facilities shall conform with the requirements of PL 91-579 (Animal Welfare Act, 1970), the amendment to PL 89-544.

3.5.2 Sanitation

3.5.2.1 Premises (building and grounds) shall be kept clean.

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3.5.2.1.1 Sanitization and sterilization of rooms and corridors.

3.5.2.1.1.1 Room and corridor floors, sinks, and pipes shall be washed with a microbicidal solution weekly. Ceilings, walls, and partitions shall be treated in a like manner at regular intervals.

3.5.2.1.1.2 After a room has been emptied of animals, all surfaces and fixed equipment shall be washed with a microbicidal solution. Portable equipment for the room shall be sanitized and sterilized, returned to the room, and the room equipment fumigated. Paraformaldehyde is recommended for this purpose.

3.5.2.2 Primary enclosure shall be cleaned and sanitized often enough to prevent an accumulation of excreta, debris, dirt and harmful contamination.

3.5.2.2.1 It shall be sanitized by washing with hot water (180°F) and soap or detergent, or by washing all soiled surfaces with a detergent solution followed by a safe and effective disinfectant, or by cleaning all soiled surfaces with live steam.

3.5.2.3 All wastes should be collected and removed regularly and frequently in a safe sanitary manner. For example: highly infectious waste should be rendered noninfectious, by autoclaving or other effective means, before removing it from the animal facility.

3.5.2.4 Most states or municipalities have statutes or ordinances controlling disposal of wastes. Compliance with these requirements is an institutional responsibility.

3.5.3 Inspection and repair

3.5.3.1 Inspection of automatic watering system.

3.5.3.1.1 Nozzles shall be inspected daily in order to assure that they are functioning properly.

3.5.3.1.2 All pipings between filters and house supply lines shall be dismounted quarterly, thoroughly cleaned, and sterilized.

3.5.3.1.3 The pressure control and supply tank for each rack or group of racks shall be cleansed and sterilized semiannually.

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Subject: MAINTENANCE OF OPTIMAL ENVIRONMENTAL CONDITIONS FOR LABORATORY ANIMALS	**Date:**	**Sheet** 5	**Of** 7

3.5.3.2 Maintain facility in good repair, to protect the animals from injury, to contain the animals, and to restrict the entrance of other animals.

3.5.3.2.1 Check for sharp corners and edges, broken wires, etc.

3.5.3.2.2 Check the walls, doors, ceilings and corners of cracks.

3.5.3.3 Check drainpipe, electric power and water supply.

3.5.3.4 Check machines such as washing machines, autoclave, etc.

4. QUALITY CONTROL

4.1 Temperature and humidity

4.1.1 The temperature and relative humidity record charts for each 24-hour period throughout each bioassay test shall be dated, signed, and filed for audit.

4.1.2 The automatic devices for recording temperature and relative humidity shall be recalibrated monthly. All pertinent data shall be entered in a bound notebook and signed by technical personnel who performed the work, and by the supervisor.

4.1.3 The alert and emergency power systems shall be tested at monthly intervals and results recorded as in 4.1.2 above.

4.2 Ventilation

4.2.1 A maintenance check on all mechanical ventilation equipment (air conditioner, blowers, fan motors, etc.) shall be made monthly.

4.2.2 Air intake and discharge filters shall be inspected at least monthly and replaced when necessary.

4.2.3 Air pressure of animal rooms with regard to entrance and egress corridors shall be checked, and adjusted if necessary each day.

4.2.4 The number of fresh-air changes per hour in animal rooms shall be monitored at least weekly and appropriate adjustments made when indicated.

4.2.5 The concentration of chemical carcinogens in discharge air must be determined as indicated in Specification No. CBO-4.

4.2.6 All data pertaining to the above shall be entered in a bound notebook and signed by personnel involved.

4.3 Lighting

4.3.1 Light intensity at cage level and at the work surface in animal examination and treatment areas shall be determined weekly and adjusted if necessary.

4.3.2 Instruments for determining light intensity shall be calibrated monthly.

4.3.3 The light cycle shall be monitored regularly and adjusted if necessary to provide diurnal cycle for the species in question.

4.3.4 The position of animal cages with respect to the light source shall be checked regularly to make certain that animals are not subjected to excessive illumination.

4.3.5 All test results and observations above shall be entered in a bound notebook and signed by personnel involved.

4.4 Noise control

4.4.1 Evaluation of noise control practices shall be included in all inspections of the laboratory animal facility and remedial measures instituted where necessary.

4.5 Facility maintenance

4.5.1 Periodic inspection of facilities.

4.5.2 Microbiologic monitoring of room surfaces, including benches, walls, and ceilings, should be done on a routine basis, but frequency depends on the desired level of protection.

4.5.3 Monitor for radiologic, toxicologic and infectious agents.

5. PACKAGING

Not applicable

6. NOTES

6.1 It should be noted that the relative humidity in the immediate vicinity of an animal in a cage (microenvironment) may be much higher than that of the animal room itself.

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6.2 Humidification may be necessary in cold months to maintain the humidity optimal for small laboratory animals.

6.3 DBA mice and certain other stocks are very susceptible to audiogenic seizures.

6.4 Convulsions have been produced even in audiogenic seizure-resistant stocks of mice by a single explosion of intense sound.

6.5 Congenital malformations have been induced in one or more animal species by audiovisual stimulation. Certain types of noise pollution could possibly alter other types of experimental results as well.

7. REFERENCE DOCUMENTS

7.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U.S. Department of Health, Education and Welfare, NIH 76-801.

7.2 Guide for the Care and Use of Laboratory Animals. 1974. U.S. Department of Health, Education and Welfare, NIH 74-23.

7.3 Long-Term Holding of Laboratory Rodents. 1976. ILAR News XIX (4), L20, L21.

7.4 The UFAW Handbook on the Care and Management of Laboratory Animals. 1972. 4th edition. UFAW Staff (eds.). Churchill Livingstone, Edinburgh and London.

7.5 Procurement Specification VII. Rodents. 1969. Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D.C.

7.6 Procurement Specification IX. Defined Laboratory Rodents and Rabbits. 1973. Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D.C.

7.7 Whitney, R. A., Jr. Physical Environment. In: Workshop on Criteria for Successful Rodent Chronic Studies, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, April 4-5, 1973.

7.8 Request for Proposal 76-S-12, Carcinogen Bioassay, NCI Program, Due date June 15, 1976, Tracor Jitco, Inc., Rockville, Maryland.

Carcinogen Bioassay Program Specification No. CBO-18

Type:	Sheet	Of
GOOD ANIMAL CARE LABORATORY PRACTICE	1	3

Subject:

SANITATION OF EQUIPMENT AND SUPPLIES FOR LABORATORY ANIMALS

Approved:	Proj.	Q.C.	Lab	Other	Date

1. SCOPE

This specification covers sanitization of cages, feeders, water bottles, and certain ancillary equipment for laboratory animals used in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

2.1	Specification No. CBM-6	Laboratory Animal Cages and Cage Filters
2.2	Specification No. CBM-8	Watering Devices for Laboratory Animals
2.3	Specification No. CBM-7	Feeders for Laboratory Animals
2.4	Specification No. CBM-9	Exposure Chambers for Inhalation Tests
2.5	Specification No. CBM-5	Racks for Laboratory Animal Cages

3. REQUIREMENTS

3.1 Cages, racks, feeders, water bottles, catch-pans, exposure chambers, and certain ancillary equipment must be sanitized at specified intervals and before reuse.

3.2 Cages shall be washed at least weekly in a machine which provides at least one cycle of 82°C (180°F) water.

3.3 Racks shall be either run through a rack washer (which has one cycle of 82°C water) every two weeks, or washed, in the sanitization area, with a suitable detergent and hosed down under high pressure.

3.4 Soiled feeders should be soaked, if necessary, and then washed in a system that uses at least one cycle of 82° C water.

3.5 If water bottles are used, bottles, bottle stoppers, and sipper tubes must be washed in water of at least 82° C temperature. Stoppers and sipper tubes must be sterilized either by germicide treatment prior to washing or by boiling after washing.

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3.6 Inhalation chambers shall be hosed down daily and sanitized weekly.

3.7 Catch-pans shall be cleaned and relined with fresh absorbent paper each day.

3.8 Mechanical washing machines with flexible time settings are highly recommended for all items where practicable.

3.8.1 Washing phase should be at least 1 1/2 minutes.

3.8.2 Recirculation rinse should be at least 1 minute.

3.8.3 Final fresh water rinse should be a minimum of 1/2 minute.

3.8.4 Weekly preventive maintenance shall be routinely practiced for washing machines. Strainers shall be cleaned at least once daily, or oftener, depending upon the work load.

3.9 Portable cleaners which dispense detergent and hot water or steam under pressure should be used, if possible, for cage racks and other pieces of equipment which are too large for the washing machine.

3.10 The pH of the detergent solution should be within the range of 10 to 12. An automatic detergent dispenser is recommended.

4. QUALITY CONTROL

4.1 Washing machine operators shall make certain that all nozzles and manifolds are constantly operative.

4.2 The pH sensing devices and heating coils shall be maintained free of any accumulation of foreign material that would impair their accuracy.

4.3 All sanitized cages, feeders, watering devices, racks, catch-pans, and exposure chambers shall be inspected for physical cleanliness prior to reuse. Unsatisfactory items shall be resanitized.

4.4 Frequency of sampling of cages and other items for microbiological monitoring of the sanitization procedure will depend upon type of decontamination and level of protection desired. No Gram-negative organisms should be detected, especially Pseudomonas spp., but spore-formers and heat-resistant non-pathogens will be found occasionally.

Detection of Gram-negative organisms should result in an immediate shutdown of washing equipment and correction of the defect or institution of better room sanitization procedures, depending on probable source.

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SANITATION OF EQUIPMENT AND SUPPLIES FOR LABORATORY ANIMALS		3	3

4.5 Cages are to be Program and Chemical specific. They shall be returned to the same chemical group and dose level to avoid possible contamination.

5. PACKAGING

Not applicable

6. NOTES

6.1 The term "sanitize" is defined as "making physically clean and removing and destroying to the maximum degree that is practical, agents injurious to health."

7. REFERENCE DOCUMENTS

7.1 Code of Federal Regulations, Title 9, Chapter 1, Subchapter A. Animal Welfare. Parts 1, 2, and 3, May, 1972.

7.2 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U.S. Department of Health, Education and Welfare, NIH 76-801.

7.3 Guide for the Care and Use of Laboratory Animals. 1974. U.S. Department of Health, Education and Welfare, NIH 74-23.

7.4 Procurement Specification IX. Defined Laboratory Rodents and Rabbits. 1973. Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D.C.

7.5 Request for Proposal 76-S-12, Carcinogen Bioassay, NCI Program, Due date June 15, 1976, Tracor Jitco, Inc., Rockville, Maryland.

Carcinogen Bioassay Program

Specification No. CBO-19

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 2

Subject:
DISINFECTION OF LABORATORY ANIMAL ROOMS

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers procedures for disinfecting laboratory animal rooms in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 If an epizootic disease occurs among animals in quarantine or on test, the area shall be disinfected before use for new animals.

3.2 Disinfectants for use in any part of the bioassay facility shall be approved by program management.

3.3 If formaldehyde gas is used, the room shall be sealed and then treated by evaporating 500 ml of formalin (37% solution of formaldehyde in water and stabilized with methyl alcohol) for each 27 m^3 of space. The temperature shall be at least 21° C (70° F) and the relative humidity 75-80% during fumigation. The exposure period shall be 24 hours.

3.4 Disinfected animal rooms shall not be reused until results of microbiological analyses indicate the absence of microorganisms pathogenic for humans and domestic animals.

4. QUALITY CONTROL

4.1 Effectiveness of the disinfection procedure shall be evaluated by sample swabbing of tables, benches, racks, walls and floor (at least one swab per area) and culturing or susceptible-host inoculation (cell cultures, embryonated eggs, or laboratory animals). Acceptable diagnostic practices of the American Society of Clinical Pathologists or an equivalent organization shall be used.

5. PACKAGING

Not applicable

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6. REFERENCE DOCUMENTS

6.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

6.2 The UFAW Handbook on the Care and Management of Laboratory Animals. 1972. 4th edition. UFAW Staff (eds.). Churchill Livingstone, Edinburgh and London.

6.3 Guide for the Care and Use of Laboratory Animals. 1974. U. S. Department of Health, Education and Welfare, NIH 74-23.

6.4 Long-Term Holding of Laboratory Rodents. 1976. ILAR News XIX (4), L20, L21.

Carcinogen Bioassay Program

Specification No. CBO-20

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 2
Subject: VERMIN CONTROL IN ANIMAL FACILITIES		

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers the control of flies, cockroaches, rodents, and like pests in quarters for animals used in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

2.1 Code of Federal Regulations, Title 9, Chapter 1, Subchapter A. Animal Welfare. Parts 1, 2, and 3, May, 1972.

3. REQUIREMENTS

3.1 A safe and effective program for the control of insects, ectoparasites, avian, and mammalian pests in and around the animal facility shall be established and maintained under the supervision of a veterinarian or other qualified person.

3.2 The building structure shall be vermin-proof with rodent barriers at all doorways.

3.3 Waste shall not be allowed to accumulate in outdoor storage areas in the vicinity of animal quarters.

3.4 Breeding sites of insects and other pests shall be eliminated or sealed with suitable materials resistant to detergents and disinfectants.

3.5 Drains in animal rooms shall be plugged.

3.6 Insulation on cage and rack washer pipes must not be exposed.

3.7 Pesticides shall not be allowed to contaminate any test animals or stored material. Pesticides ("bait") may be used inside the building only in raceways and hallways but not in animal rooms.

3.8 Strict sanitary practices on a regular basis shall be an integral part of the vermin control program.

3.8.1 All animal rooms and other areas where food and bedding particles may accumulate shall be wet-mopped daily.

3.8.2 Storage items shall be moved once each week and the floor beneath thoroughly cleaned and mopped.

Carcinogen Bioassay Program Specification No. CBO-20

Subject:	Date:	Sheet	Of
VERMIN CONTROL IN ANIMAL FACILITIES		2	2

3.9 A system for controlling escaped rodents by means of suitable traps shall be maintained at all times.

4. QUALITY CONTROL

4.1 The animal facility shall be inspected weekly for the presence or evidence of vermin and remedial measures instituted if necessary. Results of inspections and remedial action taken shall be recorded in a bound notebook, dated, and signed by inspector and supervisor.

5. PACKAGING

Not applicable

6. NOTES

6.1 Wild rodents and other vermin carry a variety of bacteria, viruses, and parasites which may be transmitted to experimental animals should they gain entrance to the facility. The population of wild rodents in the vicinity of animal buildings should be reduced or eliminated.

7. REFERENCE DOCUMENTS

7.1 Guide for the Care and Use of Laboratory Animals. 1974. U.S. Department of Health, Education and Welfare, NIH 74-23.

7.2 The UFAW Handbook on the Care and Management of Laboratory Animals. 1972. 4th edition. UFAW Staff (eds.). Churchill Livingstone, Edinburgh and London.

7.3 Rodents. Standards and Guidelines for the Breeding, Care, and Management of Laboratory Animals. National Research Council, National Academy of Sciences, Washington, D.C., 1969.

7.4 Long-Term Holding of Laboratory Rodents. 1976. ILAR NEWS XIX (4), L20, L21.

Carcinogen Bioassay Program Specification No. CBO-21

Type:	Sheet	Of
GOOD ANIMAL CARE LABORATORY PRACTICE	1	2

Subject:

SACRIFICE OF LABORATORY ANIMALS (EUTHANASIA)

Approved:	Proj.	Q.C.	Lab	Other	Date

1. SCOPE

This specification covers the requirements of euthanasia and the operational steps of euthanasia by physical and chemical methods.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Procedure requirements for euthanasia.

3.1.1 Euthanasia should be performed by trained persons in accordance with institutional policies and applicable laws.

3.1.2 The choice of method should depend on the species of animal and the project for which the animal was used.

3.1.3 The method of euthanasia should not interfere with any postmortem examinations or determinations to be performed.

3.2 Operational steps of euthanasia. Mice and rats can be killed by two main methods: the physical method and the chemical method.

3.2.1 Physical methods. Some manual dexterity is essential for this method. When done by skilled operator, apprehension on the part of the animal is minimal, death is quick and suffering slight.

3.2.1.1 Cervical dislocation. The animal is held by its tail and placed on a surface that it can grip, when it will stretch itself out so that a pencil or similar object can be placed firmly across the neck. A sharp pull on the tail will then dislocate the neck and kill at once.

3.2.1.2 Stunning. Concussion is the cause of death here.

3.2.1.2.1 Hold the animal's head downwards and strike very hard behind the ears with a stout wooden stick or stunner.

Subject: SACRIFICE OF LABORATORY ANIMALS (EUTHANASIA)	Date:	Sheet 2	Of 2

3.2.1.2.2 Hold the animal firmly, belly upwards, and strike the back of the head very hard against a hard horizontal surface such as a sink or a table.

3.2.2 Chemical Methods.

3.2.2.1 Carbon dioxide euthanasia, using a specially designed cabinet is the recommended method.

3.2.2.2 Sodium pentobarbital, injected intraperitoneally at three times the anesthetic dose can also be used.

3.2.2.3 The use of ether in an uncrowded chamber has been done before.

3.2.2.4 The use of nitrogen is not recommended.

4. QUALITY CONTROL

Not applicable

5. PACKAGING

Not applicable

6. NOTES

6.1 EUTHANASIA means the humane destruction of an animal accomplished by a method which produces instantaneous unconsciousness and immediate death without visible evidence of pain or distress, or a method that utilizes anesthesia produced by an agent which causes painless loss of consciousness, and death following such loss of consciousness.

7. REFERENCE DOCUMENTS

7.1 Guide for the Care and Use of Laboratory Animals. 1974. U.S. Department of Health, Education and Welfare, NIH 74-23.

7.2 The UFAW Handbook on the Care and Management of Laboratory Animals. 1972. 4th edition. UFAW Staff (eds.). Churchill Livingstone, Edinburgh and London.

Carcinogen Bioassay Program Specification No. CBO-22

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 2
Subject: DISPOSAL OF DEAD OR SACRIFICED ANIMALS AND TISSUES		

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers disposal of dead or sacrificed animals and tissues involved in the Carcinogen Bioassay Program.

2. APPLICABLE DOCUMENTS

2.1 Code of Federal Regulations, Title 9, Chapter 1, Subchapter A. Animal Welfare. Parts 1, 2, and 3, May, 1972.

2.2 Specification No CBT-2	Gross Necropsy of Carcinogen Bioassay Animals
2.3 Specification No. CBT-5	Histopathologic Examination of Carcinogen Bioassay Animals
2.4 Specification No. CBO-4	NCI Safety Regulations for Research Involving Chemical Carcinogens

3. REQUIREMENTS

3.1 All procedures involved in disposal of dead animals and tissues shall be in conformance with Federal, State, and local laws and regulations pertaining to pollution control and protection of the environment.

3.2 All animals which die or are sacrificed in repeated-dose, subchronic, and chronic studies shall be subjected to gross necropsy (unless cannibalism or autolysis make the animal unfit for all or part of the examination).

3.3 Carcasses of animals may be discarded immediately after necropsy and fixation of all tissues required for histopathologic examination.

3.4 Contaminated wastes, cleaning devices, and animal carcasses shall be transported to the disposal area in a closed impermeable container and disposed of by methods approved by the Office of Research Safety.

3.5 Refrigerated storage shall be available for holding dead animals until necropsy. The area shall be separate from all other cold storage and shall be used exlusively for refuse storage. The temperature shall be kept below 7° C (45°F). The animals shall not be frozen.

Subject:	Date:	Sheet	Of
DISPOSAL OF DEAD OR SACRIFICED ANIMALS AND TISSUES		2	2

3.6 All dead animals shall be subjected to full gross and histologic examination in accordance with CBT-2 Gross Necropsy Examination and CBT-5 Histopathologic Examination. Carcasses may be discarded immediately following necropsy and fixation of all required tissues.

4. QUALITY CONTROL

4.1 Supervisory personnel shall monitor the disposal of all dead and sacrificed animals and tissues to make certain that all procedures are in accord with Federal, State, and local laws as well as with regulations of the Office of Research Safety.

4.2 Containers, liners, covers, etc., used in storage and disposal of sacrificed animals and tissues shall be inspected during operations to maintain conformance with safety regulations.

5. PACKAGING

Not Applicable.

6. REFERENCE DOCUMENTS

6.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

6.2 Request for Proposal 76-S-12, Carcinogen Bioassay, NCI Program, Due date June 15, 1976, Tracor Jitco, Inc., Rockville, Maryland.

Carcinogen Bioassay Program Specification No. CBO-23

Type: GOOD ANIMAL CARE LABORATORY PRACTICE	**Sheet** 1	**Of** 2

Subject: DISPOSAL OF RADIOACTIVE WASTES ASSOCIATED WITH LABORATORY ANIMAL EXPERIMENTS

Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers the operations of the disposal of radioactive wastes associated with laboratory animal experiments and the product requirements involved in these procedures.

2. DEFINITIONS

Not applicable here.

3. REQUIREMENTS

3.1 Procedure requirements.

3.1.1 Radioactive waste must be disposed of in accordance with applicable regulations and license.

3.1.2 Set up regular schedule for the elimination of radioactive waste.

3.1.3 Use leakproof disposable liners in waste cans for disposal of radioactive waste.

3.2 Product requirement.

3.2.1 Facilities must be provided for holding radioactive waste.

3.2.1.1 The storage area for radioactive waste should be physically separated from other storage facilities and animals.

3.2.1.2 In some instances, the ordinary storage facilities may be used for holding the waste, if properly monitored, until all radioactivity has decayed.

3.2.2 Special shielding of the storage area may be required.

3.2.3 Cage washing equipment should be of type capable of decontaminating cage and accessories without accumulating radioactive waste. Machines should not recirculate the wash solution.

Carcinogen Bioassay Program Specification No. CBO-23

Subject: DISPOSAL OF RADIOACTIVE WASTES ASSOCIATED WITH LABORATORY ANIMAL EXPERIMENTS	**Date:**	**Sheet** 2	**Of** 2

4. QUALITY CONTROL

4.1 Institute a system of equipment monitoring.

5. REFERENCE DOCUMENTS

5.1 Guide for the Care and Use of Laboratory Animals. 1974. U. S. Department of Health, Education and Welfare, NIH 74-23.

Carcinogen Bioassay Program Specification No. CBO-24

Type: STANDARD OPERATING PROCEDURE				**Sheet** 1	**Of** 3
Subject: DISPOSITION OF CARCINOGEN BIOASSAY PATHOLOGY MATERIAL					
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers pathology materials to be submitted to program management as well as materials to be retained by the bioassay laboratory.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 The following material shall be submitted to program management:

3.1.1 One hematoxylin and eosin (H & E) stained slide and the tissue block representative of each different neoplasm or treatment-related lesion from each chemical test group shall be sent to program management as soon as the test is completed or earlier, if possible, or if consultation is desired. These samples shall then be submitted to the Tumor Pathology Section for record file. There should be no more than 10 slides and blocks per compound.

3.1.2 All pathologic specimens are the property of the sponsor and must be submitted to the program management upon request and automatically at end of the subcontract.

3.2 The subcontractor shall retain all wet tissues, blocks, and slides of all animals (test, vehicle controls, untreated controls) in a vermin-proof, temperature-controlled area until termination of the bioassay investigation.

3.3 All animal tissues shall be retained in formalin until program management directs their disposal. However, tissues of repeated-dose animals may be discarded after the subchronic test has been started. Subchronic and chronic residual material shall be retained and shipped to the repository when directed.

Carcinogen Bioassay Program Specification No. CBO-24

Subject:	**Date:**	**Sheet**	**Of**
DISPOSITION OF CARCINOGEN BIOASSAY PATHOLOGY MATERIAL		2	3

4. QUALITY CONTROL

4.1 Pathologist-in-Charge and Histology Supervisor shall be responsible for making certain that all required slides and tissue specimens as indicated in Sections 3 and 5 are submitted for storage and shipment.

4.2 Responsible bioassay personnel shall see to it that all materials to be retained by the laboratory are packaged and stored as indicated in Sections 3 and 5.

4.3 The Shipping Department Supervisor shall make certain that all tissue specimens, blocks, and slides to be sent to program management are packaged and shipped in accordance with Sections 3 and 5 requirements. All clearance to ship and shipment papers shall be dated, signed, and filed for audit.

5. PACKAGING

5.1 At termination of the bioassay investigation, residue of all chronic animals, and those of sub-chronic animals which were subjected to histopathologic diagnosis, shall be organized, packed, marked, and shipped to program management, after obtaining clearance.

5.2 Wet tissues (residue from harvested tissues, not carcasses) shall be stored in two plastic bags (one inside the other) and organized by histology number. A label (permanent ink) containing name of subcontractor and histology number shall be placed between the two bags. Bags shall then be packed in double-wall cardboard boxes (350 lb-test) and labelled on one end as follows:

(a) Name of subcontractor
(b) Subcontract number
(c) Chemical number
(d) Animal Group number(s)
(e) Histology numbers in that box

Boxes shall be sealed shut with shipping tape, bound with filament tape, and shipped to program management upon receipt of clearance to ship.

5.3 Blocks shall be resealed with paraffin, permanently labelled with name of subcontractor and histology number and organized according to the histo number. For shipment, blocks shall be placed in single-wall cardboard boxes (approximately 80 block size) which shall then be packed into double-wall cardboard containers (350 lb-test) approximately 16" x 18" x 7 1/2" and

Subject: DISPOSITION OF CARCINOGEN BIOASSAY PATHOLOGY MATERIAL	**Date:**	**Sheet** 3	**Of** 3

sealed with pressure tape and bound with filament tape. One end shall show the information given in 5.2 above.

5.4 Slides shall be organized by histology number and placed in plastic boxes which shall then be packaged in a 350 lb-test cardboard box, separated by abundant packing material, for shipment to program management.

5.4.1 Each plastic slide box shall be labelled with name of subcontractor and range of histology numbers.

5.4.2 Each cardboard shipping box shall contain a packing list with name of subcontractor, number of slide boxes, and cross-reference information (animal number, histology number, chemical number) for complete identification of the contents.

5.4.3 A master log (reproduction acceptable) of histology number assignments shall be sent to program management along with the first shipment of slides. This log shall be updated as required.

5.4.4 Plastic slide boxes will be sent to the bioassay laboratory upon request. All other supplies for shipment of residual material to the repository shall be obtained by the subcontractor.

6. REFERENCE DOCUMENTS

6.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

6.2 Request for Proposal 76-S-12, NCI Carcinogen Bioassay Program, Due Date June 15, 1976, Tracor Jitco, Inc., Rockville, Maryland.

Carcinogen Bioassay Program

Specification No. CBO-25

Type:	Sheet	Of
DATA RECORDS AND REPORTS SPECIFICATION	1	5

Subject:

REQUIRED INFORMATION

Approved:	Proj.	Q.C.	Lab	Other	Date

1. SCOPE

This specification lists the minimum required information for the report on bioassay study.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 Devise a plan for the collection of required information before the start of the bioassay study.

3.2 All information and data pertaining to the bioassay shall be completely and accurately recorded on a current basis.

3.3 Minimum required information includes:

GENERAL:
1. Outline of the bioassay study
2. Bioassay study number in the investigator's file
3. Names of the investigators responsible for the bioassay study, including histopathological diagnoses
4. Name of the bioassay laboratory

CHEMICAL (TEST AGENT):
1. Name, chemical abstract number, NCI number
2. Name, synonyms
3. Formula
4. Source (generic)
5. Manufacturer
6. Batch number
7. Date(s) when received
8. Storage (before its reception)
9. Physical state and other characteristics
10. Melting point
11. Solubility
12. Criteria of purity
13. Impurities (generic)

Carcinogen Bioassay Program Specification No. CBO-25

Subject:	Date:	Sheet	Of
REQUIRED INFORMATION		2	5

14. Methods of synthesis
15. Storage conditions and dates
16. Other

PREPARATION:
1. Chemical(s): name, data sheet number
2. Vehicle(s): name, data sheet number
3. Preparation and concentration
4. Methods of preparation
5. Amount prepared each time
6. Frequency of preparation
7. Physical state
8. pH
9. Stability and decomposition
10. Storage
11. Date(s) prepared
12. Other

ANIMALS:
1. Species
2. Strain and subline
3. Initial number by sex (male and female)
4. Date(s) of birth (male and female)
5. Source
    a. Own colony (give reference)
    b. Other
6. Breeding
    a. Inbred
    b. Random
    c. Outbred
    d. Other
7. Disease control
    a. Specific pathogen-free
    b. Germ-free
    c. Conventional
    d. Vaccinated
8. Distribution in groups
    a. Pooled at weaning
    b. Random
    c. Random tables
    d. Littermates
    e. Other
9. Other experimental groups included in the same distribution
10. Initial number per cage
11. Divided by sex
12. Age when obtained from Animal House

Subject: REQUIRED INFORMATION	**Date:**	**Sheet** 3	**Of** 5

13. Maintenance (general conditions)
    a. Own standard (give reference)
    b. Special
14. Cages
15. Bedding
16. Room temperature (range)
17. Light
    a. Source
    b. Time cycle
18. Diet
    a. Type
    b. Source
19. Amount of diet
    a. Ad libitum
    b. Measured
20. Water
    a. Tap
    b. Other
21. Amount of water
    a. Ad libitum
    b. Measured
22. Other or special conditions

TREATMENT:
1. Special pretreatment conditions
2. Treatment multiplicity
    a. Single type
    b. Combined

Item 3-14 should apply for each treatment

3. Preparation administered
4. Dose per administration
    a. Volume
    b. Weight
    c. How measured
5. Route
6. Site
7. Methods and instruments used
    a. Surgical procedures
    b. Anesthesia
    c. Sterility
8. Frequency of administration
9. Total number of doses
10. Total dose given
11. Total time of treatment
12. Date treatment started
13. Date treatment ended
14. Others

Carcinogen Bioassay Program

Specification No. CBO-23

Subject:	Date:	Sheet	Of
REQUIRED INFORMATION		4	5

PLAN OF OBSERVATIONS:
1. Age of animals at start of experiment
2. Weight of animals at start of experiment
3. Duration of experiment
    - a. Lifespan
    - b. Interruptions (from when to where and why)
4. Frequency of checking
5. Frequency of weighing
6. Frequency of charting
7. Frequency of measuring consumption of:
    - a. Feed
    - b. Water
8. Other observations
9. Autopsies
    - a. On all animals
    - b. With the exception of:
        - (1) decomposed animals
        - (2) lost animals
        - (3) other
10. Autopsy examinations
    - a. Complete
    - b. Except cranial cavity
    - c. Other exceptions
11. Histology
    - a. All tumors (note exceptions)
    - b. Other tissues
12. Other pathological observations

CONTROLS:
1. List:
    - a. Each group
    - b. Selection

VARIATIONS:
1. List protocol additions or change

REPORTS:
1. Animal groups
    - a. Body weight curves
2. Individual animals
    - a. Identification number
    - b. Mode of death (died or sacrificed)
    - c. Time of death (in days or weeks of age, or time from start of bioassay study)
    - d. Diagnosis of tumors found at necropsy and other pertinent pathology
    - e. Indication if necropsy not done (as in decomposition) and animal is considered lost from the study

Carcinogen Bioassay Program Specification No. CBO-25

Subject: REQUIRED INFORMATION	**Date:**	**Sheet** 5	**Of** 5

4. QUALITY CONTROL

Proof-read the report to ensure that no required information is missing.

5. PACKAGING

Not applicable here.

6. REFERENCE DOCUMENTS

6.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

Carcinogen Bioassay Program　Specification No. CBO-26

Type: DATA RECORDS AND REPORTS SPECIFICATIONS				**Sheet** 1	**Of** 2
Subject: NCI CARCINOGEN BIOASSAY DATA SYSTEM (CBDS)					
Approved:	**Proj.**	**Q.C.**	**Lab**	**Other**	**Date**

1. SCOPE

This specification covers the purpose and requirements of a log book. Carcinogenesis Bioassay Data System (CBDS) by The National Cancer Institute is described, including its quality control processes.

2. APPLICABLE DOCUMENTS

None

3. REQUIREMENTS

3.1 The purpose of a log book is to enable ease, accuracy, and completeness in recording and retrieving data, since the preparation of reports require the extraction, review, consolidation, and tabulation of data from the log books.

3.2 The National Cancer Institute has developed a computerized system to collect, retrieve, tabulate, and analyze bioassay test data.

3.1 This CBDS also serves to manage and monitor the status and progress of individual bioassay studies as well as to summarize the total effort of the CBP.

3.2 Data input is through a series of forms (see specification on "CBDS Data Forms") submitted to the CBP where they are processed for entry into a computer.

3.3 The Systemalized Nomenclature of Pathology (SNOP) is used to code the pathology results collected on individual animals.

3.4 Data output is available as a series of standard or special reports and tables presenting the data in the following way:

- a. Bioassay studies underway within the total program or within a particular contract effort
- b. An individual bioassay study
- c. Special pathology reports
- d. Survival and weight curves

Carcinogen Bioassay Program
Specification No. CBO-26

Subject:	**Date:**	**Sheet**	**Of**
DATA RECORDS AND REPORTS SPECIFICATIONS		2	2

e. Selected analysis of the test data

4. QUALITY CONTROL

4.1 Closely monitor data before their entrance into the CBDS.

4.2 Periodically hold special training classes for data technicians.

4.3 Data forms must be checked before being translated into machine-readable format and entered into the system, microfilmed, and stored as microfiche for future reference.

4.4 Data are only available for selective or complete recall after errors have been edited by computer.

5. PACKAGING

Not applicable here.

6. REFERENCE DOCUMENTS

6.1 Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U. S. Department of Health, Education and Welfare, NIH 76-801.

APPENDIX C

QUALITY CONTROL SURVEILLANCE CHECK LIST FOR MICROBIOLOGY

The following appendices, 1, 1A, 1B, 1C, and 2, are reprinted with permission from the publisher. They are from "Functional Quality Control" by R. C. Bartlett, in Quality Control in Microbiology edited by J. E. Prier, J. T. Bartola and H. Friedman, and published by the University Park Press, Baltimore, Maryland 21202, in 1973.

APPENDIX 1

Surveillance Report

Personnel responsible for conducting surveillance activities are defined in the surveillance schedule. If any item is not monitored as scheduled, an explanation must be provided on a Surveillance Report supplement, sheet A. Any error or deficiency found must be reported.

Verification of the recording of error in the Surveillance Report will be made by circling unacceptable observations on laboratory work sheets along with the insertion of the letters SR, the date, and the recorder's initials. A complete description of the error is made on sheet B. This must include a description of the action taken to resolve the problem, whether a solution was found or the matter is pending. A and B sheets must be submitted to the supervisor for compilation into the monthly surveillance report to the director. The supervisor will review all pending deficiencies reported during the previous month. The ones that continue in pending status are submitted with the next surveillance report.

The following symbols have been appended to each item to assist other laboratories in attaching priorities to the progressive development of surveillance programs: *, low priority; **, medium priority; and ***, high priority.

Abbreviations: BAP, blood agar plates; CTA, cystine Trypticase agar; DNase, desoxyribonuclease; G-N, gram-negative; MR-VP, methyl red/Voges-Proskaver; ONPG, orthonitrophenylgalactosidase; PAD, phenylalanine deaminase; PSE, Pfizer selective Enterococcus; RA, rheumatoid arthritis (RA factor); SAE, Society of Automotive Engineers; SIM, sulfide indole motility; TSA, tryptic soy agar; TSN, tryptic sulfite neomysin; XL, xylose lysine; XLD, xylose lysine decarboxylase.

Suppliers: ^{a}, BBL, Division of BioQuest, Becton, Dickinson and Co. (Cockeysville, Md.); ^{b}, Clay Adams (New York, N.Y.); ^{c}, Difco Laboratories (Detroit, Mich.); ^{d}, Ortho Diagnostics (Raritan, N.J.); ^{e}, Pfizer, Diagnostics Division (New York, N.Y.); ^{f}, Roche Diagnostics, Division of Hoffman-LaRoche Inc. (Nutley, N.J.); ^{g}, Statens Seruminstitut (Copenhagen, Denmark); ^{h}, Warnpole Laboratories (Stamford, Conn.).

APPENDIX 1A

METHODS (responsibility for surveillance is indicated under each item)	Frequency of Surveillance	Standards to be Monitored
Procedure Book ***	3 Months	All sections of the procedure book are indexed by author. Each section will be monitored every 3 months by the author. The supervisor will reassign sections when employees terminate. Corrections will be submitted to the division secretary for retyping, copying, and distribution to established procedure book locations. Deficient procedures will be listed as pending until corrections are distributed.
Evening Shift Personnel Review ***	1–6 Months	Review of procedures with evening personnel by area assistant supervisor. Procedures to be reviewed and frequency will be established by supervisor.
Night Shift Personnel Review ***	1–6 Months	Review of procedures with night shift personnel by assistant supervisor. Procedures to be reviewed and frequency will be established by supervisor.
Proficiency Testing Specimens *	Monthly	Supervisor receives samples, submits them to area assistant supervisors, and later checks all forms before mailing by the deadline. Assistant supervisor reports results at weekly meeting. Supervisor receives critiques and discusses any discrepancies with area assistant supervisors. Surveillance is applied to completion of analyses and mailing of reports before the deadline, review of critiques, maintenance of specimen viability, and resolution of discrepancies. To be conducted by area assistant supervisor.

Appendix 1A—*Continued*

	Frequency of Surveillance	Standards to be Monitored
Cultures Submitted to Reference Laboratory ***	Monthly	Area assistant supervisor is responsible for preparing and sending samples to reference laboratory, copying lab slips and putting them in proper book, maintaining samples for retesting in case of discrepancies, resolving any discrepancies, and discussing results with supervisor. Surveillance is applied to resolution of discrepancies, and maintenance of specimen viability.
Blind Unknowns **		
Bacteriology	Monthly	In duplicate by supervisor.
Clinical microscopy	Daily	Submitted by area assistant supervisor.
Serology	Monthly	Submitted and results reviewed by supervisor.
Mycology	2 Months	
Mycobacterium	Monthly	
Fluorescence microscopy	Monthly	
		Blind unknowns are submitted by nondiagnostic supervisor. Results on all of the above are correlated and discussed with diagnostic supervisor and area assistant supervisors. Diagnostic supervisor then discusses results.
BACTERIOLOGY (surveillance conducted by bacteriology assistant supervisor)		
General		
Antimicrobial susceptibility testing disc method		
Materials ***	Each batch of medium	Control strains tested with each batch of media: (a) coordinate media preparation for testing, (b) coordinate stock and use of antibiotics in test, (c) maintain control strains, and (d) calculate precision and error. Control reporting of results on drugs yielding unacceptable results.

Appendix 1A—Continued

	Frequency of Surveillance	Standards to be Monitored
Personnel **	3 Months	Test each worker for technique, precision, and accuracy.
Antimicrobial susceptibility testing—tube dilution—penicillin **	Monthly or as requested	Monitor technique and procedure with stock strain of Enterococcus of known minimum inhibitory concentration. Results must be 3–12 units/ml.
Frequently Used Reagents *		
Antisera for bacteriological identification	6 Months	Culture antisera in use for 6 months. Rotate all antisera in use, according to inventory. Test new antisera with known cultures.
Escherichia coli, A,B, and C		
Haemophilus influenzae, A and B		
Shigella A–D		
Salmonella A–I, vi, polyvalent		
Alkalescens dispar		
Bethesda Ballerup		
Arizona		
Stains		
Gram stain reagents	Monthly	Fresh stock dated. Discard remaining portions every 6 months.
Flagella stain	Monthly	Fresh stock dated. Discard remaining portions yearly. Combined stain made fresh with each use and discarded after use.
Hydrogen peroxide	Monthly	Replace with fresh stock every 2 months.
Equipment (surveillance to be conducted by assistant supervisor in each area)		
Refrigerators ***		
MB 4338 Foster 2 door	Weekly	2–8°C
MB 4348 Jewett		
MB 4347 Foster 6 door		
Foster 5 door		
Automatic alarms	6 Months	

Appendix 1A—*Continued*

	Frequency of Surveillance	Standards to be Monitored
Freezer ***		
MB 4307 Dillon Lilly	Weekly	2–8°C
Incubators ***		
MB 4339 Labline 1d	Weekly	
MB 4371 Labline 2d	Weekly	
Temperatures	Weekly	35 ± 1°C
Water	Weekly	Pan full
Fan motors	Weekly	Running smoothly
	6 Months	MB 4371 oil with 20 SAE
Vents	6 Months	Change glasswool filter
MB 4380 Labline CO_2		
Temperature	Weekly	35 ± 1°C
Water	Weekly	Bubbler system full
Fans	Weekly	Running smoothly
Fan motor	6 Months	Oil with 20 SAE
CO_2 tanks	Weekly	Not empty
surge tank	Weekly	12 pounds during surge
flow tank	Weekly	12 pounds at 0.3 liters/min
CO_2 concentration	Yearly	5–10% Monitor for 48 hr with medical gas analyzer (pulmonary lab)

Appendix 1A—Continued

	Frequency of Surveillance	Standards to be Monitored
Humidity	Monthly Weekly	Submit syringe full of gas 60–80%
Water baths and heating blocs ***		
MB Hospital control		
MB 4319 variable	Weekly	54–56°C
MB 4369 TSN bloc		
MB 4368 variable		45–47°C
Inoculating loops *	Monthly	3-mm diameter; replace as needed
Inoculating wire *	Monthly	5–8 cm; replace as needed
Safety hood ***	6 Months	Face velocity, 50 feet/min
	Monthly	Filter pressure, 0.5–1.5 cm
	3 Months	Wash interior with germicidal cleaner
Air conditioner *	Monthly	Change filter (engineering department)
Grinding motor *	6 Months 6 Months	Oiled Fan belt, wear, tension
Microscopes *	Monthly	General inspection
Anaerobic jars ***	Daily Daily Daily	*Clostridium novyi* control Change catalyst Anaerobic indicator

Appendix 1A–Continued

	Frequency of Surveillance	Standards to be Monitored
IMMUNOLOGY (surveillance to be conducted by area assistant supervisor)		
Procedures ***		
Antistreptolysin "O" titer *Brucella* Rheumatoid factor Thyroglobulin antibody Typhoid O VDRL Antinuclear factor *E. coli* *Neisseria gonorrhoeae* *Streptococcus* Rhodamine-auramine staining of mycobacteria	As performed or once a month	Prepare and distribute positive and negative controls, approve results before reporting. Record control data graphically.
VDRL (Venereal Disease Research Laboratory) ***	Weekly	Monitor proper use and recording of controls. Graphically record comparison of state laboratory and Hartford Hospital results. Resolve results differing by more than two tubes.
Rhodamine-auramine stain **	Monthly	Fresh stock dated. Discard remaining portion every 2 months.
Refrigerators ***		
MG 4337	Weekly; automatic alarms every 6 months.	
Waterbaths ***		
MB 4384 VDRL	Weekly	55–57°C
MB 4390 miscellaneous	Weekly	36–38°C

Appendix 1A—*Continued*

	Frequency of Surveillance	Standards to be Monitored
VDRL Rotators *		
MB 4335 FA	Weekly	Lubricate bearings
MB 4387 VDRL	Weekly	
VDRL rotators *	Weekly	180 rpm (VDRL only)
Inoculating loops *	Replace monthly or as needed	5-mm diameter loop
Safety hood ***	6 Months	Face velocity, 50–200 fpm
Microscopes *	6 Months	General inspection
Fluorolume illuminator *	As bulb change needed	Bulb should be changed every 150 hr, with maintenance as follows: Check reflector adjustments and clean; check bulb adjustments and fan motor; Clean exciter filter and window assembly.
CLINICAL MICROSCOPY (surveillance to be conducted by area assistant supervisor)		
Biologicals **		
Occult blood test	Daily	Affirm 4+ positive reaction to 1:1,000 aqueous solution blood
(Benzidine dihydrochloride: H_2O_2) Hydrogen peroxide		Replace with fresh stock every 2 months.
Wright stain White and red blood cell diluting fluids and stains Trichome stain	Monthly	Replace with fresh stock every 2 months.
Sudan		Discard remainder and prepare fresh yearly.

Appendix 1A—*Continued*

Equipment	Frequency of Surveillance	Standards to be Monitored
Refrigerator *** MB 4372	Weekly 6 Months	2–8°C Test automatic alarms.
Refractometer *** MB 4388 MB 4390	3 Months	Calibrate with H_2O to 1.000
Glassware *	Monthly	Discard chipped glassware
Fume hood *	3 Months	Wash with germicidal cleaner
Microscopes *	Monthly	General inspection
MEDIA ROOM(surveillance to be conducted by media room personnel)		
Equipment		
Refrigerator *** MB 4394	Weekly	2–8°C
Hot air oven ***	Daily	Record each run; must be 155–165°C
	3 Months	Sterility check (spore strips)
Autoclaves ***	3 Months Weekly	Sterility check (spore solution) Check gaskets

Appendix 1A—Continued

	Frequency of Surveillance	Standards to be Monitored
Glassware *	Monthly	Discard chipped glassware
Balance *	3 Months	Calibrate
	6 Months	Clean and general maintenance (described in instruction manual)
MYCOLOGY (surveillance to be conducted by area assistant supervisor)		
Equipment		
Water baths ***	Weekly	
Incubator *** MB 4330	Weekly	35 ± 1°C
Safety hood ***	6 Months	Face velocity 50–200 fpm
	3 Months	Wash with germicidal cleaner
Microscope *	Monthly	General inspection
MISCELLANEOUS (surveillance to be conducted by designated supervisor)		
Equipment		
Vacuum pump (for lyophilization)*		Before use and when in continual use the following are performed:
	3 Months	0.5-inch belt tension
	3 Months	Oil level
	6 Months	Oil parts
	Yearly	Change oil, order new oil

Appendix 1A—Continued

	Frequency of Surveillance	Standards to be Monitored
Vacuum pump (for air sampler)*	6 Months	Oil as indicated
Pipettes *	As received	Check calibration
Thermometers *	As received	Calibrate with National Bureau of Standards calibrated thermometer
MEDIA (surveillance functions to be delegated by supervisor)		
Autoclave Cycles***	Each batch	
Test tubes, 16 × 150 mm with 10 ml of agar 1,000-ml volumes in flask 2,000-ml volumes in flask		Recording of min of exposure to 121°C (minimum/maximum) 12/24 20/24 30/72
pH***	Each batch	Must be ± 0.2 from recommended pH of manufacturer (except [illegible], 7.3–7.4)
Storage*** Plates Not bagged: 2-week duration Bagged: 10-week duration	Weekly	2–4°C

Appendix 1A—*Continued*

	Frequency of Surveillance	Standards to be Monitored
Tubes, sponge-plugged		2–8°C
Not bagged: 2-week duration		
Bagged: 2-month duration		
Tubes, screw-capped		2–8°C
Not bagged: 2-month duration		
Bagged: 4-month duration		
Exceptions		
Cysteine Trypticase agar (CTA) sugars: 2-week duration		Room temperature
Indole nitrate: bagged, 2-week duration		Room temperature
Thioglycolate: stored in darkness, 4 months		Room temperature
Depth of Plates**		
Plates		3 Mm (except Mueller-Hinton, 4–6 mm)
Tubes	Each batch	
Agar		Length of slant equal to length of butt
Broths		Specified in reagents and media section
Sterility Check***		
Broth (blood)	Each batch	3 Tubes, incubated 48 hr
Plates (BAP and chocolate only)	Each batch	5% Of plates incubated 48 hr (chocolate agar, 72 hr)
Testing with Stock Cultures***	Each batch* or each lot +	Proper hemolysis Correct color reactions Inhibitory or selective properties

APPENDIX 1B: Testing with Stock Cultures

Item	Control Organisms	Acceptable Results
Plates BAP*	Group A β-hemolytic *Streptococcus*	Good β-hemolysis
	Streptococcus viridans	Good α-hemolysis
	Blank	Sterile
Biplates*	See BAP and MacConkey	See BAP and MacConkey
Brain heart infusion agar*	*Cryptococcus* sp.	Growth
Brain heart infusion agar and chloramphenicol	*Nocardia* sp.	Growth
	Escherichia coli	No growth
Casein plates*	*Streptomyces* sp.	Hydrolysis
	Nocardia asteroides	No hydrolysis
Chocolate*	*Hemophilus influenzae*	Good growth
	Neisseria gonorrhoeae	Good growth (48 hr)
Martin-Lester*	*Neisseria gonorrhoeae*	Good growth (48 hr)
	Staphylococcus aureus	Inhibition
	Escherichia coli	Inhibition
	Proteus mirabilis	Inhibition of swarming
	Candida albicans	Inhibition
MacConkey+	*Escherichia coli*	Lactose-positive; correct morphology
	Shigella flexneri (B)	Colorless colonies
	Enterococcus	Inhibition
Mycobiotic agar*	*Candida albicans*	Growth
	Aspergillus	No growth
	Escherichia coli	No growth

Appendix 1B—*Continued*

Item	Control Organisms	Acceptable Results
Mueller-Hinton*	See surveillance of disk susceptibility (Appendix 2A, Bacteriology, General)	Disc
Phenylethyl alcohol agar (PEA)*[c]	*Staphylococcus aureus*	Growth
	Escherichia coli	Inhibition (tiny colonies)
	Pseudomonas aeruginosa	Inhibition (tiny colonies)
Potato dextrose agar*	*Trichophyton rubrum*	Growth
Rice agar*	*Candida albicans*	Chlamydospores (72 hr)
	Candida krusei	No chlamydospores (72 hr)
Rodac*[a]	*Flavobacterium*	Growth
Sabouraud's dextrose*	*Candida albicans*	Growth
Sabouraud's dextrose* agar and chloramphenicol	*Aspergillus* sp.	Growth
	Escherichia coli	No growth
Trypticase soy agar+	*Staphylococcus aureus*	Growth
	Streptococcus viridans	Growth
Tyrosine plates*	*Streptomyces* sp.	Positive
	Nocardia asteroides	Negative
Xanthine plates*	*Streptomyces* sp.	Positive
	Nocardia asteroides	Negative
XLD	*Salmonella typhimurium*	Black colonies
	Shigella flexneri (B)	Red (colorless) colonies
	Escherichia coli	Yellow colonies

Abbreviations: *, each batch; ', each lot; +, positive; 0, negative; D, delayed; A, acid; ALK, alkaline; AG, acid and gas; NC, no change.

Appendix 1B—*Continued*

Item	Control Organisms	Acceptable Results
Tubes		
Christensen's urea*	*Proteus mirabilis*	+
	Klebsiella pneumoniae	D
	Escherichia coli	0
Christensen's urea* slants (mycology version)	*Torulopsis* sp.	Positive
	Candida sp.	Negative
DNase*	*Serratia*	+
	Staphylococcus epidermidis	0
Middlebrook 7H-10*	*Nocardia*	Positive
	Mycobacterium tuberculosis	Positive
Pseudosel*		Growth and pigment
	Pseudomonas aeruginosa	+ +
	Escherichia coli	0 0
SIM+		H_2S indole motility
	Proteus mirabilis	+ 0 +
	Klebsiella pneumoniae	0 + +
	Escharichia coli	0 + +
Simmon's citrate+	*Klebsiella pneumoniae*	+
	Escherichia coli	0
Stuart's transport+	*Neisserria gonorrhoeae*	
	22°C, 6-hr transfer	Growth (48 hr)
	2–8°C, 6-hr transfer	Growth (48 hr)

Appendix 1B—*Continued*

Item	Control Organisms	Acceptable Results
	Clostridium novyi	
	22°C, 6-hr transfer	Growth (24 hr)
	2–8°C, 6-hr transfer	Growth (24 hr)
TSA+	*Staphylococcus aureus*	Growth
	Streptococcus viridans	Growth
Triple sugar iron (TSI)+	*Escherichia coli*	A/AG
	Salmonella typhimurium	ALK/AG H_2S+
	Proteus rettgeri	ALK/A
	Pseudomonas aeruginosa	ALK/NC
TSN+	*Clostridium perfringens*	+
	Clostridium novyi	0
Indole nitrate*	Indole	
	Escherichia coli	+
	Klebsiella pneumoniae	0
	Nitrate	
	Staphylococcus aureus	+
	Hérellea sp.	0
	Pseudomonas aeruginosa	+g
Gelatin* (mycology version)	*Cladosporium*	Positive
	P. pedrosoi	Negative
Rice grains*	*Microsporum canis*	Positive
	Microsporum audouini	Negative
Bile* (sodium desoxycholate)	*Streptococcus pneumoniae*	+
	Streptococcus viridans	0
Blood broth*	*Hemophilus influenzae*	Growth (24 hr)
	Neisseria meningitidis	Growth (24 hr)

Appendix 1B—*Continued*

Item	Control Organisms	Acceptable Results
Brain heart infusion agar*	*Cryptococcus albidus*	Growth
Brain heart infusion agar and chloramphenicol*	*Cryptococcus albidus*	Growth
Decarboxylase*		
Control*	*Proteus mirabilis*	0
	Klebsiella pneumoniae	0
	Pseudomonas aeruginosa	NC
With ornithine*	*Proteus mirabilis*	+
	Klebsiella pneumoniae	0
	Pseudomonas aeruginosa	NC
With lysine*	*Escherichia coli*	+
	Enterobacter cloacae	0
	Pseudomonas aeruginosa	NC
Gelatin*	*Pesudomonas aeruginosa*	+
	Herellea	0
Gluconate+	*Pseudomonas aeruginosa*	+
	Herellea	0
G-N Broth+	*E. coli:Shigella* = 1:1	
	6-hr transfer	Recovery of *Shigella*
	18-hr transfer	Recovery of *Shigella* Inhibition of *E. coli*
	E. coli:Shigella = 2:1	
	6-hr transfer	Recovery of *Shigella*
	18-hr transfer	Recovery of *Shigella*

Appendix 1B—*Continued*

Item	Control Organisms	Acceptable Results
Inositol*	*Trichosporon verrucosum*	Positive
Malonate+	*Klebsiella pneumoniae*	+
	Escherichia coli	0
MR-VP+	*Escherichia coli*	+/0
	Klebsiella pneumoniae	0/+
ONPG+	*Escherichia coli*	+
	Proteus mirabilis	0
PAD*	*Proteus mirabilis*	+
	Escherichia coli	0
PSE+[c]	*Enterococcus*	+
	Group D not *Enterococcus*	+
	Listeria (48 hr)	+
	Group A β-hemolytic *Streptococcus*	0
Rabbit plasma+	*Staphylococcus aureus*	+
	Staphylococcus epidermidis	0
6.5% Salt broth+	*Enterococcus*	+
	Group D not *Enterococcus*	0
	Staphylococcus aureus	+
	Group A β-hemolytic *Streptococcus*	0
Sucrose assimilation*	*Candida albicans*	+
Thiamine*	*Trichophyton tonsurans*	+
	Trichophyton menta	0
Thioglycolate+	*Clostridium novyi*	Growth
	Streptococcus viridans	Growth

Appendix 1B—*Continued*

Item	Control Organisms			Acceptable Results	
Tryptic soy broth+		*Streptococcus viridans*		Growth (24 hr)	
Urea broth* (Rustigian and Stewart)		*Nocardia brasiliens*		+	
Oxidation-fermentation (OF) sugars*	*Proteus rettgeri* (lactose-fermentative)	*Proteus morganii*	*Pseudomonas aeruginosa*	*Pseudomonas maltophilia*	Arizona
Glucose	A	A	A/0	0	A
Sucrose	A	0	0	0	0
Maltose	0	0	0	A	A
Xylose	0	0	A	0	A
Arabinose	*Serratia*	0			
	Enterobacter	A			
CTA sugars*[d]	*Neisseria gonorrhoeae*	*Neisseria meningitidis*	*Neisseria sicca*		*Neisseria catarrhalis*
Glucose	A	A	A		0
Maltose	0	A	A		0
Sucrose	0	0	A		0
Lactose	0	0	0		0

APPENDIX: 1C

Inventory of reagents and biologicals**

Storage code: A, room temperature; B, 2–8°C; C, cool; D, dry; E, protect from light; F, dangerous to handle for one or more of the following: 1, poison, 2, caustic, 3, corrosive, 4. avoid contact (absorbed through skin, strong oxidizing agent, etc.), 5, should not be inhaled, 6, volatile, 7, extremely toxic, or 8, carcinogen; G, freeze; H, dessicated; NS, none stated or none found in reference.

Expiration code: M, month; W, week; Y, year; S, stated on product; D, does not apply; NS, none stated or none found in reference.

References

Merck Index of Chemicals and Drugs. 1960. Merck & Co., Rahway, N.J.

BBL Manual of Products and Laboratory Procedures. 1968. 5th Ed. BBL-BioQuest, Division of Becton, Dickinson and Co., Cockeysville, Md.

Personal communication, David A. Power, Ph. D., Manager of Marketing Communications, BioQuest, Division of Becton, Dickinson and Co., Cockeysville, Md.

BIOLOGICALS-IMMUNOLOGY (surveillance to be conducted by area assistant [illegible]ervisor)

Item	Expiration		Storage and/or Precautions		Surveillance Interval
	Opened	Closed	Opened	Closed	
Albumin, bovine	2M	3Y	B	B	Weekly
ANF conjugate	6M	1Y	G	B	Weekly
ASO buffer	1M	2Y or S	B	B	Weekly
ASO reagent	10 min	2Y or S	B	B	Weekly
ASO standard	2M	2Y or S	G	B	Weekly
Brucella	2M	S	B	B	Weekly
E. coli A(conjugate) (Difco)^c	2M	1Y	B	B	Weekly
E. coli A(serological)	6M	2Y	B	B	Weekly
E. coli B(conjugate) (Difco)^c	2M	1Y	B	B	Weekly
E. coli B(serological)	6M	2Y	B	B	Weekly

Appendix 1C–*Continued*

Item	Expiration		Storage		Surveillance Interval
	Opened	Closed	Opened	Closed	
E. coli C(conjugate)	2M	1Y	B	B	Weekly
E. coli C(serological)	6M	2Y	B	B	Weekly
Hemagglutination buffer	1M	2Y	B	B	Weekly
Monospot kit[d]	1M	S	B	B	Weekly
Neisseria gonorrhoeae conjugate	2M	1Y	B	B	Weekly
Phosphate-buffered saline (PBS)	1M	NS	B	A	Weekly
Pregnosticon (slide)–50 test[j]	2W	S	B	B	Weekly
Pregnosticon (slide)–10 test[j]	1M	S	B	B	Weekly
RA buffer	1M	S	B	B	Weekly
RA latex 0.81	1M	1Y	B	B	Weekly
RA plasma fraction II	1M	1Y	B	B	Weekly
RA test kit	1M	S	B	B	Weekly
Rabbit globulin; fluorescent antibody	2M	1Y	B	B	Weekly
Rabbit plasma, discard remainder after use	2W	1Y	B	B	Weekly
Streptococcus conjugate	2M	4M			Weekly
Streptococcus controls	2M	1Y	A	B	Weekly
Streptozyme kit[h]	1M	S	B	B	Weekly
Thyroid kit	1M	S	B	B	Weekly
Typhoid "O"	2W	S	B	B	Weekly
Typhoid "O" and *Brucella* control (neg.)	3M	NS	G	B	Weekly

Appendix 1C– ***Continued***

Item	Expiration		Storage		Surveillance Interval
	Opened	Closed	Opened	Closed	
Typhoid "O" and *Brucella* control (pos.)	3M	S	G	B	Weekly
VDRL antigen and buffer	2W	1Y	A	E	Weekly
VDRL control sera	3M	S	G	B	Weekly
BIOLOGICALS-BACTERIOLOGY (surveillance to be conducted by area assistant supervisor)					
Ampicillin	6M	S	G,H	G,H	Weekly
Arizona (open when needed)					
diphasic	1Y	S	B	B	Weekly
monophasic	1Y	S	B	B	Weekly
Carbenicillin	6M	S	G,H	G,H	Weekly
Cephalothin	6M	S	G,H	B,H	Weekly
Chloramphenicol	6M	S	G,H	B,H	Weekly
Colistin	6M	S	G,H	B,H	Weekly
Erythromycin	6M	S	G,H	B,H	Weekly
Gentamicin sulfate	6M	S	G,H	B,H	Weekly
Hemophilus type A	6M	S	B	B	Weekly
Hemophilus type B	6M	S	B	B	Weekly
Horse serum	6M	1Y	0–4°C	0–4°C	Monthly
Kanamycin	6M	S	G,H	B,H	Weekly
Lincomycin hydrochloride	6M	S	G,H	B,H	Weekly
Methicillin	6M	S	G,H	G,H	Weekly
Nalidixic acid	6M	S	B,H	B,H	Weekly
Nitrofurantoin	6M	S	B,H	B,H	Weekly
Omni serum for *Streptococcus pneumoniae*[g]	1Y	2Y	0–5°C	0–5°C	Weekly
Oxytetracycline	6M	S	G,H	B,H	Weekly
Penicillin (disc)	6M	S	G,H	G,H	Weekly

Appendix 1C–*Continued*

Item	Expiration		Storage and/or Precautions		Surveillance Interval
	Opened	Closed	Opened	Closed	
Penicillin (powder, standard)	**6M**	**S**	**G,H**	**G,H**	**Weekly**
Penicillinase	**1Y**	**5Y**	**B**	**B**	**Weekly**
Rabbit plasma, normal-coagulase	**2W**	**3Y**	**G**	**B**	**Weekly**
***Salmonella* O group A**	**6M**	**2Y**	**B**	**B**	**Weekly**
***Salmonella* O group B**	**6M**	**2Y**	**B**	**B**	**Weekly**
***Salmonella* O group C1**	**6M**	**2Y**	**B**	**B**	**Weekly**
***Salmonella* O group C2**	**6M**	**2Y**	**B**	**B**	**Weekly**
***Salmonella* O group D**	**6M**	**2Y**	**B**	**B**	**Weekly**
***Salmonella* O group E**	**6M**	**2Y**	**B**	**B**	**Weekly**
***Salmonella* O group F**	**6M**	**2Y**	**B**	**B**	**Weekly**
Salmonella O group G	6M	2Y	B	B	Weekly
Salmonella O group H	6M	2Y	B	B	Weekly
Salmonella O group I	6M	2Y	B	B	Weekly
Salmonella O polyvalent	6M	1Y	B	B	Weekly
Salmonella vi	6M	2Y	B	B	Weekly
Shigella group A	6M	2Y	B	B	Wwwkly
Shigella group B	6M	2Y	B	B	Weekly
Shigella group C	yM	2Y	B	B	Weekly
Shigella group D	6M	2Y	B	B	Weekly
Shigella–Alkalescens dispar	6M	2Y	B	B	Weekly
Streptomycin	6M	S	G,H	B,H	Weekly
Sulfisoxizole (Gantrisin)	6M	S	B,H	B,H	Weekly

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Appendix 1C–*Continued*

Item	Expiration		Storage and/or Precautions		Surveillance Interval
	Opened	Closed	Opened	Closed	
Tetracycline	6M	S	G,H	B,H	Weekly
Vancomycin hydrochloride	6M	S	B,H	B,H	Weekly

STOCK CARBOHYDRATES-SURVEILLANCE

Item	Expiration Opened	Expiration Closed	Storage Opened	Storage Closed	Surveillance Interval
Frequently used stored in crystal form					
Arabinose	6M	2Y	C,D	C,D	Weekly
Dextrose	6M	2Y	C,D	C,D	Weekly
Lactose	6M	2Y	C,D	C,D	Weekly
Maltose	6M	2Y	C,D	C,D	Weekly
Sucrose	6M	2Y	C,D	C,D	Weekly
D-Xylose	6M	2Y	C,D	C,D	Weekly
Infrequently used stored in disc form					
Adonitol	6M	2Y	C,D	C,D	Weekly
Dulcitol	6M	2Y	C,D	C,D	Weekly
Galactose	6M	2Y	C,D	C,D	Weekly
Inositol	6M	2Y	C,D	C,D	Weekly
Inulin	6M	2Y	C,D	C,D	Weekly
Levulose	6M	2Y	B,H	B,A	Monthly
Mannitol	6M	2Y	B,H	B,A	Monthly
Mannose	6M	2Y	B,H	B,A	Monthly
Raffinose	6M	2Y	B,H	B,A	Monthly
Rhamnose	6M	2Y	B,H	B,A	Monthly
Salicin	6M	2Y	B,H	B,A	Monthly
Sorbitol	6M	2Y	B,H	B,A	Monthly
Trehalose	6M	2Y	B,H	B,A	Monthly

Appendix 1C—*Continued*

STOCK CHEMICALS

Item	Expiration	Storage and/or Precautions	Surveillance Interval
Acetic acid, glacial	NS	F_1, 4	Monthly
N-Acetyl-*l*-cystein	NS	B_1	Monthly
p-Aminodimethylaniline oxalate	NS	D	Monthly
Ammonium sulfate powder	NS		Monthly
Aniline	NS	F_1, E	Monthly
Auramine O	5Y	D	Monthly
Barium chloride	NS	F_1	Monthly
Barium sulfate	3M prepared	NS	Monthly
Benzidine dihydrochloride	6M prepared	NS,F4,8	Monthly
Bromthymol blue	5Y		Monthly
Calcium chloride	NS	C,D	Monthly
China blue powder (Poirier's blue)	NS	D	Monthly
Chlorazol black E	5Y	D	Monthly
Crystal violet	5Y	D	Monthly
Cysteine hydrochloride	NS	D	Monthly
p-Dimethylaminobenzaldehyde	NS	E	Monthly
Dimethylsulfoxide	NS	NS	Monthly
Eosin Y	5Y	D	Monthly
Ether		F5,6	Monthly
Ethyl alcohol	3M opened NS sealed	D	
Ferric ammonium citrate	NS	E	Monthly
Ferric chloride	NS	E	Monthly
Fuchsin, acid	5Y	D	Monthly
Fuchsin, basic	5Y	D	Monthly
Glycerine	NS	F4	Monthly
Gramercy indicator	NS	NS	Monthly
Hemin	NS	NS	Monthly

Appendix 1C– *Continued*

Item	Expiration	Storage and/or Precautions	Surveillance Interval
Hydrogen chloride, conc. (chemistry)	NS	F4,5	Monthly
Hydrogen peroxide 3%	NS	E,C,F2	Monthly
Iodine crystals	NS	NS	Monthly
Lactic acid	NS	NS	Monthly
Magnesium sulfate	NS	D	Monthly
Malachite green	5Y	D	Monthly
Menadione	NS	E	Monthly
Mercuric chloride	NS	F1	Monthly
Merthiolate	NS		Monthly
Methyl red	5Y	D	Monthly
Methylene blue	5Y	D,F1	Monthly
Naphthol	NS	E	Monthly
Naphthylamine	NS	F4,8	Monthly
Orthonitrophenyl-*βd*-galactopyranoside	NS	NS	Monthly
Oxgall	1Y	C,D	Monthly
Periodic acid	NS	NS	Monthly
Phenol (crystals)	NS	D,E,F4	Monthly
Phenolphthalein diphosphate	NS	F4	Monthly
Phenyl red	5Y	D	Monthly
Phenylalanine	NS	NS	Monthly
Potassium alum	NS	NS	Monthly
Potassium ferrocyanide	working soln.–immediately	NS	Monthly
Potassium hydroxide	NS	F2	Monthly
Potassium iodide	NS	NS	Monthly
Potassium permanganate	5Y	D	Monthly
Potassium phosphate dibasic	NS	NS	Monthly
Potassium phosphate monobasic	NS	NS	Monthly
Rhodamine O	5Y	D,F1	Monthly
Safranin	5Y	D	Monthly
Sedi stain[b]	5Y	D	Monthly
Sodium *m*-bisulfate	NS	NS	Monthly
Sodium carbonate	NS	D,E,F1,4	Monthly
Sodium chloride	NS	NS	Monthly

Appendix 1C– *Continued*

Item	Expiration	Storage and/or Precautions	Surveillance Interval
Sodium citrate	NS	NS	Monthly
Sodium desoxycholate	NS	NS	Monthly
Sodium hydroxide	NS	F2	Monthly
Sodium phosphate monobasic	NS	NS	Monthly
Sodium phosphate tribasic	NS	NS	Monthly
Sodium succinate	NS	NS	Monthly
Sodium thiosulfate	NS	NS	Monthly
Sudan III	5Y	D	Monthly
Sulfanilic acid	NS	NS	Monthly
Sulfosalicylic acid	NS	D,E	Monthly
Tannic acid	NS	E	Monthly
N,N,N,N,-Tetramethyl-*p*-phenylene diamine monohydrochloride	NS	F1,4	Monthly
Thiamine	NS	NS	Monthly
Thymol (Merck)	NS	NS	Monthly
Toluidine blue	5Y	D,F1	Monthly
Trichrome	5Y	D	Monthly
Triphenyltetrazolium chloride	NS	NS	Monthly
Trisodium citrate			Monthly
Trypan blue	5Y	D	Monthly
l-Tryptophane	NS	NS	Monthly
Tyrosine	NS	NS	Monthly
Xanthine	NS	F1	Monthly
Zinc dust metal	NS	F5	Monthly
Zinc sulfate			

Appendix 1C– *Continued*

MEDIA (surveillance to be conducted by media room personnel under direction of supervisor)

	Expiration Date		
Product	Opened	Closed	Storage
Agar-agar	1Y	3Y	C,D
Anaerobic agar	1Y	3Y	C,D
Brain heart infusion agar	1Y	3Y	C,D
Brain heart infusion broth	1Y	3Y	C,D
Brilliant green agar	1Y	3Y	C,D
Bordet-Gengou	1Y	3Y	C,D
Chloramphenicol	6M	S	B,D
Clostrisel agar[a]	1Y	2Y	C,D
Columbia agar base	1Y	3Y	C,D
Cooked meat medium	1Y	3Y	C,D
Cystine Trypticase agar (CTA) medium[a]	1Y	3Y	C,D
DNase test medium	1Y	3Y	C,D
Entamoeba medium	1Y	3Y	C,D
Fletcher medium base	1Y	3Y	C,D
GC medium base	1Y	3Y	C,D
Gelatin	1Y	3Y	C,D
GN broth	1Y	3Y	C,D
Indole nitrate broth	1Y	3Y	C,D
Litmus milk	1Y	2Y	C,D
Littman Oxgall (prepared)	D	S	B
Loeffler blood serum	1Y	2Y	C,D
Loeffler (prepared)	D	1Y	B
Lysine iron agar	1Y	2Y	C,D
MacConkey	1Y	3Y	C,D
Malonate	1Y	3Y	C,D
Moeller decarboxylase	1Y	3Y	B
MR-VP	1Y	3Y	C,D
Mueller-Hinton agar	1Y	3Y	C,D
Mycobactosel agar[a]	D	1Y	B
Mycobactosel L-J agar[a]	D	1Y	B
Mycobiotic[c]	1Y	2Y	C,D
Neopeptone[c]	1Y	3Y	C,D
Oxidation-fermentation basal medium	1Y	3Y	C,D
Pfizer selective Enterococcus	1Y	3Y	C,D
Phenol red broth base	1Y	3Y	C,D
Phenylethyl alcohol agar (PEA)	1Y	3Y	B
Phytone peptone[a]	1Y	3Y	C,D
Plate count agar	1Y	3Y	C,D
Pseudosel[a]	1Y	3Y	C,D
Purple milk	1Y	3Y	C,D
Resazurin	1Y	3Y	C,D
Rice extract agar	1Y	3Y	C,D
Sabouraud's agar (modified)	1Y	3Y	C,D
SIM	1Y	3Y	C,D
Simmon's citrate	1Y	3Y	C,D
Skim milk powder	1Y	3Y	C,D
Spirit blue agar	1Y	3Y	C,D
Stuart's transport	1Y	3Y	B
Thiosulfate citrate bile salts (TCBS) medium	1Y	3Y	C,D
Tellurite glycine agar base	1Y	3Y	C,D
Thiogel medium[a]	1Y	3Y	C,D
Thioglycolate with dextrose and Eh indicator	1Y	3Y	C,D
Thioglycolate without dextrose and Eh indicator	1Y	3Y	C,D

Appendix 1C–*Continued*

Product	Expiration Date		Storage
	Opened	Closed	
Thioglycolate fluid	1Y	3Y	C,D
Todd-Hewitt	1Y	3Y	C,D
Triple sugar iron (TSI) agar	1Y	3Y	C,D
Tripticase soy agar with lethicin and polysorbate 80 [a]	1Y	3Y	B
Trypticase soy agar [a]	1Y	3Y	B
Trypticase soy broth [a]	1Y	3Y	C,D
TSN [a]	1Y	2Y	C,D
Tuberculosis (TB) niacin test	1Y	1Y	C,D
Urea agar base	1Y	2Y	B
Urea broth	1Y		
XL agar base	1Y	2Y	C,D
Yeast extract	1Y	3Y	C,D

APPENDIX 2:

QUALITY CONTROL PROGRAM MONTHLY REPORT[a]

Division of Microbiology
Department of Pathology
Hartford Hospital
Date ______

	Conducted as scheduled (if NO give explanation sheet A)		Deficiency observed (if YES give explanation sheet B)	
	Yes	No	Yes	No
Methods				
Procedure book	( )	( )	( )	( )
Evening shift review	( )	( )	( )	( )
Night shift review	( )	( )	( )	( )
Referee samples CAP	( )	( )	( )	( )
CDC	( )	( )	( )	( )
Check samples CAP	( )	( )	( )	( )
ASCP	( )	( )	( )	( )
Evaluation (Conn. State Department of Health)	( )	( )	( )	( )
Reference cultures	( )	( )	( )	( )
Blind unknowns				
Clinical microscopy	( )	( )	( )	( )
Bacteriology	( )	( )	( )	( )
Serology	( )	( )	( )	( )
Mycology	( )	( )	( )	( )
Parasitology	( )	( )	( )	( )
Mycobacterium	( )	( )	( )	( )
Fluorescent miscroscopy	( )	( )	( )	( )
Susceptibility (disc control)	( )	( )	( )	( )
Antimicrobial susceptibility Tube dilution	( )	( )	( )	( )
Biological materials				
Antisera (bacteriological)	( )	( )	( )	( )
Antigens (serology)	( )	( )	( )	( )
Fluorescent controls	( )	( )	( )	( )
Reagents	( )	( )	( )	( )
Equipment				
Refrigerator	( )	( )	( )	( )
Freezers	( )	( )	( )	( )
Incubators	( )	( )	( )	( )

Appendix 2–*Continued*

	Conducted as scheduled (If NO give explanation sheet A)		Deficiency observed (If YES give explanation sheet B)	
	Yes	No	Yes	No
Water baths and heating blocks	( )	( )	( )	( )
Hot air oven	( )	( )	( )	( )
Autoclaves	( )	( )	( )	( )
VDRL rotator	( )	( )	( )	( )
Inoculating loops	( )	( )	( )	( )
Inoculating wires	( )	( )	( )	( )
Safety hood (small)	( )	( )	( )	( )
Safety hood (large)	( )	( )	( )	( )
Vacuum pump (lyophile)	( )	( )	( )	( )
Vacuum pump	( )	( )	( )	( )
Refractometer	( )	( )	( )	( )
Glassware	( )	( )	( )	( )
Detergent	( )	( )	( )	( )
Grinding motor	( )	( )	( )	( )
Media room balance	( )	( )	( )	( )
Microscopes	( )	( )	( )	( )
Pipettes	( )	( )	( )	( )
Thermometer	( )	( )	( )	( )
Inventory				
Biologicals–serology				
ASO buffer	( )	( )	( )	( )
ASO reagent	( )	( )	( )	( )
ASO standard	( )	( )	( )	( )
Brucella	( )	( )	( )	( )
Cold agglutinin cells	( )	( )	( )	( )
Fever control (negative)	( )	( )	( )	( )
Fever control (positive)	( )	( )	( )	( )
Hemagglutination buffer	( )	( )	( )	( )
Monospot kit	( )	( )	( )	( )
Pregnosticon (slide)	( )	( )	( )	( )
Pregnosticon (tube)	( )	( )	( )	( )
RA buffer	( )	( )	( )	( )
RA latex 0.81	( )	( )	( )	( )
RA plasma fraction II	( )	( )	( )	( )
RA test kit	( )	( )	( )	( )
Syphilitic serum (4+)	( )	( )	( )	( )
Thyroid kit	( )	( )	( )	( )
Typhoid "O"	( )	( )	( )	( )
VDRL antigen and buffer	( )	( )	( )	( )

Appendix 2–*Continued*

	Conducted as scheduled (if NO give explanation sheet A)		Deficiency observed (if YES give explanation sheet B)	
	Yes	No	Yes	No
Biologicals				
Ampicillin	( )	( )	( )	( )
ANF conjugate	( )	( )	( )	( )
Arizona diphasic	( )	( )	( )	( )
Arizona monophasic	( )	( )	( )	( )
Carbenicillin	( )	( )	( )	( )
Cephalothin	( )	( )	( )	( )
Chloramphenicol	( )	( )	( )	( )
Colistin	( )	( )	( )	( )
Erythromycin	( )	( )	( )	( )
E. coli A (conjugate) (Difco)	( )	( )	( )	( )
E. coli A (serological)	( )	( )	( )	( )
E. coli B (conjugate) ODifco)	( )	( )	( )	( )
E. coli B (serological)	( )	( )	( )	( )
E. coli C (conjugate)	( )	( )	( )	( )
E. coli C (serological)	( )	( )	( )	( )
Gentamicin sulfate	( )	( )	( )	( )
Hemophilus type A	( )	( )	( )	( )
Hemophilus type B	( )	( )	( )	( )
Kanamycin	( )	( )	( )	( )
Lincomycin hydrochloride	( )	( )	( )	( )
Methicillin	( )	( )	( )	( )
Naladixic acid	( )	( )	( )	( )
Neisseria gonorrhoeae conjugate	( )	( )	( )	( )
Nitrofurantoin	( )	( )	( )	( )
Omni serum for *Pneumococcus*	( )	( )	( )	( )
Oxytetracycline	( )	( )	( )	( )
Penicillin (disc)	( )	( )	( )	( )

[a] Abbreviations: CAP, College of American Pathologists; CDC, Center for Disease Control; ASCP, American Society of Clinical Pathologists; ASO, antistreptolysin 0; RA, rheumatoid arthritis; ANF, antinuclear factor.

Sheet A

Form used to report items not monitored as scheduled. Reason must be given along with corrective action.

Sheet A: Use for each surveillance item not conducted as scheduled. Use additional sheets if necessary.

Item ____

Reason not conducted:

Item ____

Reason not conducted:

Item ____

Reason not conducted:

Sheet B1

Form used to record detection of deficiency through monitoring. Description must be thorough and include corrective action. Ultimate resolution of problem must be defined.

Sheet B1: Use for items when surveillance reveals deficiency.

Item ______________________________

Deficienty observed: Date ______________________________

Describe:

____

____

____

____

Date corrective action taken ______________________________

Nature of corrective action:

____

____

____

____

Was deficiency corrected? _____Yes _____No Date______________________________

If not corrected, Explain:

Further surveillance needed ☐

Sheet B2:

Item	Date observed	Deficiency observed and action taken	Results of action taken and conclusion	Procedural changes	Was Deficiency Corrected		Further surveillance needed
					Yes	No	

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